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PATENT COOPERATION TREAT .

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From the INTERNATIONAL BUREAU

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To:

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Francisca, Hendrika, Maria
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PAYS-BAS

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

Date of mailing (day/month/year) 04 August 2000 (04.08.00)	
Applicant's or agent's file reference L/UV24/102	**IMPORTANT NOTIFICATION**
International application No. PCT/EP99/00748	**International filing date** (day/month/year) 04 February 1999 (04.02.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
LEUVEN RESEARCH & DEVELOPMENT VZW Groot Begijnhof Benedenstraat 60 B-3000 Leuven Belgium	BE	BE
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
THROMB-X N.V. Leopold I straat 1 bus 21 B-3000 Leuven Belgium	BE	BE
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer N. Wagner Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

003449716

ATENT COOPERATION TRI Y

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 28 October 1999 (28.10.99)	
International application No. PCT/EP99/00748	**Applicant's or agent's file reference** L/UV24/102
International filing date (day/month/year) 04 February 1999 (04.02.99)	**Priority date** (day/month/year) 04 February 1998 (04.02.98)
Applicant COLLEN, Désiré, José	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

02 September 1999 (02.09.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was
[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer F. Baechler Telephone No.: (41-22) 338.83.38

ATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
L/UV24/102		
International application No. PCT/EP99/00748	International filing date *(day/month/year)* 04/02/1999	Priority date *(day/month/year)* 04/02/1998
International Patent Classification (IPC) or national classification and IPC C12N15/31		
Applicant LEUVEN RESEARCH & DEVELOPMENT VZW et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☒ Priority
   III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☒ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/09/1999	26.05.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Morawetz, R Telephone No. +49 89 2399 8155

I. Basis of the r port

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-86	as originally filed			

Claims, No.:

1-30	as received on	01/05/2000	with letter of	01/05/2000

Drawings, sheets:

1/5-5/5	as originally filed			

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

see separate sheet

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has

been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant dat .

3. Additional observations, if necessary:

s e separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 1-5, 9-13, 26 and in part 6, 14-17, 27-30.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 1-5, 9-13, 26 and in part 6, 14-17, 27-30.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	6 (part), 7, 8, 14 (part),15-17 (part), 18-25
	No:	Claims	14 (part), 27- 30
Inventive step (IS)	Yes:	Claims	6 (part*), 14 (part*), 7, 8, 15 (part*) -17 (part*), 18-25
	No:	Claims	6 (part'), 14 (part'), 15 (part') -17 (part')
Industrial applicability (IA)	Yes:	Claims	6 (part), 7, 8, 14 (part)-17 (part), 18-25, 27 (part)-30 (part)
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separat sheet

Re Item I

Basis

1. The amendments filed with the letter dated 01.05.2000 appear to be allowable under Article 34(2)(b) PCT. The amendments concerned are the following:

1.1. Claim 6 corresponds to original claim 7 of which not novel SakSTAR variants have been omitted.
Claims 7 and 8 correspond to original claims 8 and 9, respectively.
Claim 14 corresponds to original claim 7.
Claims 15-25 correspond to original claims 12-22, respectively. In claims 18-25 the reference to table 20 has been substituted by the SakSTAR indication corresponding to the code used in Table 20.
Claims 27-30 correspond to original claims 24-27, respectively.

2. The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established (claims 1-5, 9-13, 26 and in part 6, 14-17, 27-30) need not be the subject of international preliminary examination (Rule 66.1 (e) PCT).

Amendments of these claims are thus of no consequence for the establishment of this report.

Applicant's attention is however drawn to the fact that introduction of the feature "provided that the other amino acid is not alanine" into claims 1-3 and of the feature "provided that at least one amino acid is replaced with an amino acid other than alanine" into claim 5 is considered to introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT.

Re Item II

Priority

1. D1 (COLLEN, D. ET AL., FIBRINOLYSIS & PROTEOLYSIS, (JUNE, 1998) VOL. 12, NO. SUPPL. 1, PP. 30. MEETING INFO.: XIVTH INTERNATIONAL CONGRESS ON FIBRINOLYSIS AND THROMBOLYSIS LJUBLJANA, SLOVENIA

JUNE 22-26, 1998), which discloses several of the presently claimed SakSTAR variants is not considered part of the prior art (Rule 64 PCT) as the relevant subject-matter of present application can validly claim the priority of EP application 98200323.8 dated 04.02.1998 (see Table 10 and Example 8).

Re Item III

Non-establishment of report with regard to novelty, inventive step or industrial applicability

1. No report will be established for claims relating to inventions in respect of which no international search report has been established (Article 34(4)(a) and Rule 66.1 (e) PCT).

Re Item IV

Lack of unity of invention

1. Rule 13 PCT stipulates that the international application shall relate to one invention only or to a group so linked as to form a single general inventive concept. Where a group of inventions is claimed in one and the same international application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding "special technical features", i.e. technical features that define a novel and inventive contribution over the prior art.

2. Reference is made to the following documents, the numbering corresponds to the listing of the documents in the international search report:

D2: COLLEN D ET AL., CIRCULATION, (1997 JAN 21), 95 (2): 455-62
D3: COLLEN D ET AL., CIRCULATION, (1996 JUL 15) 94 (2): 207-16
D4: COLLEN D ET AL., CIRCULATION, (1996 JUL 15) 94 (2): 197-206
D5: COLLEN D ET AL., CIRCULATION, (1997 JAN 21) 95 (2): 463-72
D6: EP-A-0 721 982 (1996-07-17)

The document D7 was cited in the application (page 86, line 10-13):

D7: INADA Y ET AL., TIBTECH 13: 86-91, (1995).

3. The only "special technical feature" common to all present claims is that they are concerned with staphylokinase derivatives having reduced immunogenicity and thrombolytic efficacy. However, staphylokinase derivatives having reduced immunogenicity and thrombolytic efficacy in general and several of the presently claimed derivatives in particular are well known in the prior art (see D2-D6). Consequently, this common feature does not unitarily link the present set of claims and in the absence of another special technical feature, the present set of claims lacks an unifying concept and each staphylokinase derivative is considered as a separate invention.

4. Although all claimed inventions have been the subject of examination, the objection regarding lack of unity may be pursued at a later time point, e.g. in the regional phase of the application.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The present application does not satisfy the criterion set forth in Article 33(2) PCT because the subject-matter of claims 14 (part), 27- 30 is not new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).

1.1. The subject-matter of claim 14(part), insofar directed to the following staphylokinase derivatives is anticipated by the prior art.

SakSTAR(K74A, E75A,R77A) has been disclosed in D2, D3, D4, D5 and D6.
SakSTAR(K35A, E75A) has been disclosed in D2.
SakSTAR(E75A) has been disclosed in D2 and D5.
SakSTAR(E80A, D82A) has been disclosed in D2, D3, D4 and D6.
SakSTAR(E80A) has been disclosed in D2.

SakSTAR(D82A) has been disclosed in D2.
SakSTAR(E75A, D82A) has been disclosed in D2.

1.2. The subject-matter of claims 27-30 is anticipated by the prior art.

The method of claim 27 is disclosed in D4 (page 199, right hand column, 2nd paragraph - page 202, left hand column, 1st paragraph), D6 (page 3, line 47-51).

The method of claim 28 is disclosed in D4 (page 199, right hand column, 2nd paragraph - page 202, left hand column, 1st paragraph).

The pharmaceutical compositions of claims 29 and 30 are anticipated by D3 (whole document), D5 (whole document) and D6 (page 3, line 55-59).

1.3. The subject-matter of claim 14 (part), insofar directed to staphylokinase derivatives other than the ones indicated in V.1.1. above and the subject-matter of claims 6 (part), 7, 8, 15-17 (part), 18-25, appears to be new in view of the available prior art.

2. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of claims 6 (part'), 14 (part'), 15 (part') -17 (part') does not involve an inventive step as defined in the regulations (Rule 65 (1)-(2) PCT).

2.1. The subject-matter of claims 6 (part') and 14 (part'), insofar directed to SakSTAR(K35A), SakSTAR(K130A) and SakSTAR(V132A) lacks an inventive step.

D4 discloses (Figure 1) the amino acid sequence of SakSTAR with indication of the charged amino acid clusters that are substituted with alanine in order to evaluate the immunoreactivity of site-specific mutants. Several of these "charged cluster-to-alanine" substitutions variants induce less antibody formation in patients than wild-type recombinant staphylokinase (SakSTAR), but their specific activities are reduced by 50%. D4 concludes that it is possible to produce engineered variants of staphylokinase that are functional but less antigenic than the wild-type molecule.

D2 and D5 study the effect of the reversal of one or more of these "charged cluster-to-alanine" substituted amino acids to the wild type residues on the ratio of activity to antigenicity.

The subject-matter of claims 6 (part') and 14 (part'), insofar directed to further alanine-substitution mutants of staphylokinase is, thus, considered obvious in view of D4 in combination with D2 or D5.

2.2. Claims 15 (part') -17 (part') concern embodiments which are familiar to the skilled person (see D2-D7). Consequently, they would only be considered inventive if they were based upon a new and inventive staphylokinase derivative. For the present claims 15 (part') -17 (part'), insofar related to the non-novel or non-inventive subject-matter of claims 6 (part') and 14 (part') this is not the case. Therefore the subject-matter of these claims is considered to be obvious.

3. The present application satisfies the criterion set forth in Article 33(3) PCT because the subject-matter of claims 6 (part*), 14 (part*), 7, 8, 15 (part*) -17 (part*), 18-25 involves an inventive step as defined in the regulations (Rule 65 (1)-(2) PCT).

3.1. Claims 6 (part*) and 14 (part*), insofar directed to further substitution mutants of staphylokinase in which amino acids were substituted with amino acids other than Ala and insofar directed to combination variants of SakSTAR(K130T, K135T) and SakSTAR(E80A, D82A, K130T, K135T) are considered to meet the requirements of the PCT with respect to inventive step.

Document D5, which is considered to represent the most relevant state of the art, discloses (whole document) staphylokinase derivatives from which the subject-matter of claims 6 (part*) and 14 (part*) differs in that it relates to staphylokinase derivatives having different amino acid substitutions.
The problem to be solved by the present invention may therefore be regarded as the provision of alternative staphylokinase derivatives having reduced antibody induction but intact thrombolytic potency.

The solution to this problem proposed in claims 6 (part*) and 14 (part*) of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:

The prior art discloses staphylokinase derivatives having reduced antibody induction but intact thrombolytic potency due to "charged cluster-to-alanine" substitutions and reversal of one or more of these substituted amino acids to the wild-type residues (see e.g. D5).

The prior art neither discloses nor suggests that staphylokinase derivatives which have substitutions other than alanine would still show reduced antibody induction while retaining intact thrombolytic potency.

3.2. Claims 7, 8 and 18-25 which relate to further combination variants of SakSTAR(K130T, K135T) and cysteine-substitutions variants of SakSTAR with reduced immunogenicity, substituted with maleimide-polyethylene glycol are also considered to meet the requirements of the PCT with respect to inventive step.

3.3. Claims 15 (part*) -17 (part*) insofar related to the novel and inventive subject-matter of claims 6 (part*), 7, 8 and 14 (part*) are considered to meet the requirements of the PCT with respect to inventive step.

Re Item VIII

Certain observations on the international application

1. Claim 15 has been interpreted as referring to claim 14 and not to claim 9.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : **C12N 15/31, C07K 14/31, A61K 38/16**	**A2**	**(11) International Publication Number:** **WO 99/40198** **(43) International Publication Date:** 12 August 1999 (12.08.99)

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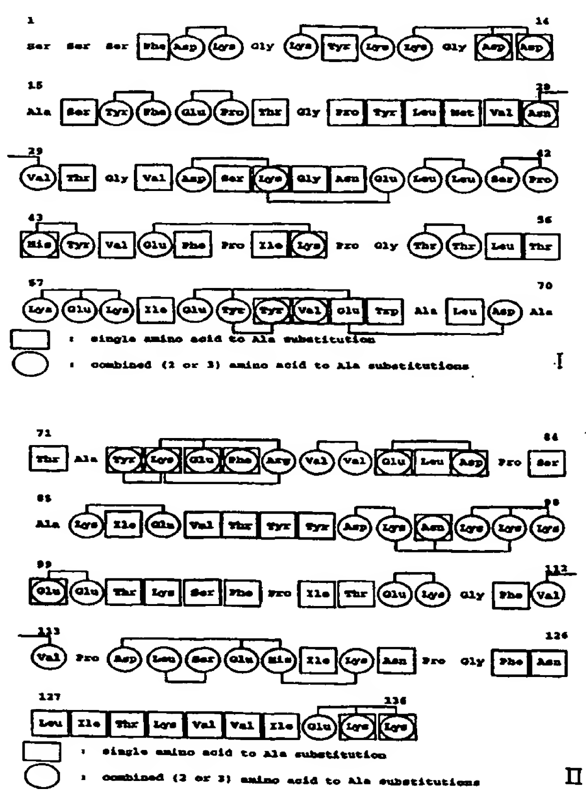
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: IDENTIFICATION, PRODUCTION AND USE OF STAPHYLOKINASE DERIVATIVES WITH REDUCED IMMUNOGENICITY AND/OR REDUCED CLEARANCE

(57) Abstract

Methods for the identification, production and use of staphylokinase derivatives characterized by a reduced immunogenicity after administration in patients and that can be administered by single intravenous bolus injection. The derivatives of the invention are obtained by preparing a DNA fragment comprising at least the part of the coding sequence of staphylokinase that provides for its biological activity; performing *in vitro* site-directed mutagenesis on the DNA fragment to replace one or more codons for wild-type amino acids by a codon for another amino acid; cloning the mutated DNA fragment in a suitable vector; transforming or transfecting a suitable host cell with the vector; culturing the host cell under conditions suitable for expressing the DNA fragment; purifying the expressed staphylokinase derivative to homogeneity and chemically modifying substituted Cys residues with thiol-directed polyethylene glycol; preferably the DNA fragment is a 453 bp EcoRI-HindIII fragment of the plasmid pMEX602sakB, (pMEX.SakSTAR), the *in vitro* site-directed mutagenesis is performed by spliced overlap extension polymerase chain reaction and the mutated DNA fragment is expressed in *E. coli* strain TG1 or WK6. The invention also relates to pharmaceutical compositions comprising at least one of the staphylokinase derivatives according to the invention together with a suitable excipient, for treatment of arterial thrombosis.



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IDENTIFICATION, PRODUCTION AND USE OF STAPHYLOKINASE DERIVATIVES WITH REDUCED IMMUNOGENICITY AND/OR REDUCED CLEARANCE

The present invention relates to new staphylokinase derivatives with reduced immunogenicity which can be administered by continuous infusion or by single intravenous bolus injection, to their identification, production and use in the treatment of arterial thrombosis and to the preparation of a pharmaceutical composition for treating arterial thrombosis. More in particular the invention relates to the use of engineered staphylokinase derivatives for the preparation of a pharmaceutical composition for treating myocardial infarction.

Staphylokinase, a protein produced by certain strains of Staphylococcus aureus, which was shown to have profibrinolytic properties more than 4 decades ago (1, 2) appears to constitute a potent thrombolytic agent in patients with acute myocardial infarction (3, 4). The staphylokinase gene has been cloned from the bacteriophages sakφC (5) and sak42D (6) as well as from the genomic DNA (sakSTAR) of a lysogenic Staphylococcus aureus strain (7). The staphylokinase gene encodes a protein of 163 amino acids, with amino acid 28 corresponding to the NH_2-terminal residue of full length mature staphylokinase (6, 8, 9). The mature protein sequence of the wild-type variant SakSTAR (9) is represented in Figure 1. Only four nucleotide differences were found in the coding regions of the sakφC, sak42D and sakSTAR genes, one of which constituted a silent mutation (6, 8, 9). In a plasma milieu, staphylokinase is able to dissolve fibrin clots without associated fibrinogen degradation (10-12). This fibrin-specificity of staphylokinase is the result of reduced inhibition by α_2-antiplasmin of plasmin.staphylokinase complex bound to fibrin, recycling of staphylokinase from the plasmin.staphylokinase complex following inhibition by

α_2-antiplasmin, and prevention of the conversion of circulating plasminogen.staphylokinase to plasmin.staphylokinase by α_2-antiplasmin (13-15). In addition staphylokinase has a weak affinity for circulating but a high affinity for fibrin-bound plasminogen (16) and staphylokinase requires NH_2-terminal processing by plasmin to display its plasminogen activating potential (17). In several experimental animal models, staphylokinase appears to be equipotent to streptokinase for the dissolution of whole blood or plasma clots, but significantly more potent for the dissolution of platelet-rich or retracted thrombi (18, 19). Staphylokinase is a heterologous protein and is immunogenic in man. The intrinsic immunogenicity of staphylokinase, like that of streptokinase, clearly hampers its unrestricted use. Not only will patients with preexisting high antibody titers be refractory to the thrombolytic effect of these agents, but allergic side effects and occasional life-threatening anaphylaxis may occur (20). Because both streptokinase and staphylokinase are heterologous proteins, it is not obvious that their immunogenicity could be reduced by protein engineering. Indeed, no successful attempts to generate active low molecular weight fragments from streptokinase have been reported. In staphylokinase, deletion of the NH_2-terminal 17 amino acids or the COOH-terminal 2 amino acids inactivates the molecule, which in addition is very sensitive to inactivation by site-specific mutagenesis (21).

It is therefore the object of the present invention to provide less immunogenic variants of staphylokinase having preferably a higher specific activity and/or lower plasma clearance and/or increased thrombolytic potency.

In the research that ultimately led to the present invention it was already found that the wild-type staphylokinase variant SakSTAR (9) contains three non-overlapping immunodominant epitopes, at least two of

which can be eliminated by specific site-directed mutagenesis, without inactivation of the molecule. This has been disclosed in EP-95200023.0 (22). These engineered staphylokinase variants are less reactive with antibodies elicited in patients treated with wild-type staphylokinase, and are significantly less immunogenic than wild-type staphylokinase, as demonstrated in rabbit and baboon models and in patients with peripheral arterial occlusion (22).

The present invention now relates to general methods for the identification, production and use of staphylokinase derivatives showing a reduced antigenicity and immunogenicity as compared to wild-type staphylokinase as well as for variants with selective derivatization with polyethylene glycol. The derivatives preferably have a higher specific activity and/or lower plasma clearance and/or increased thrombolytic potency. The derivatives have essentially the amino acid sequence of wild-type staphylokinase or modified versions thereof and essentially intact biological activities, but have a reduced reactivity with a panel of murine monoclonal antibodies and/or with antibodies induced in patients by treatment with wild-type SakSTAR. The polyethylene glycol substituted ("pegylated") variants have reduced plasma clearances rendering them particularly suited for use by single intravenous bolus administration. Instead of PEG other pharmaceutically acceptable macromolecules can be used.

More in particular, the invention provides for staphylokinase derivatives SakSTAR(K35X,G36X,E65X,K74X, E80X,D82X,K102X,E108X,K109X,K121X,K130X, K135X,K136X, +137X) having the amino acid sequence as depicted in figure 1 in which the amino acids Lys in position 35, Gly in position 36, Glu in position 65, Lys in position 74, Glu in position 80, Asp in position 82, Lys in position 102, Glu in position 108, Lys in position 109, Lys in position 121, Lys in position 130, Lys in position 135 and/or Lys in position 136 have been replaced with other amino acids and/or in which one amino acid has been added at the COOH-terminus, thus altering the immunogenicity

after administration in patients, without markedly reducing the specific activity.

Further preferred embodiments of the invention are staphylokinase derivatives listed in Tables 1, 3, 4, 5, 6, 7, 8, 13, 19 and 20, having the amino acid sequence as depicted in figure 1 in which the indicated amino acids have been replaced by other amino acids thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase, without reducing the specific activity.

Derivatives in which the specific activity is increased and the immunogenicity is decreased are the following:

SakSTAR(K74A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E75A), SakSTAR(E80A,D82A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(S34G,G36R, H43R), SakSTAR(K35A), SakSTAR(E80A), SakSTAR(D82A,S84A), SakSTAR(T90A), SakSTAR(Y92A), SakSTAR(K130A), SakSTAR(V132A), SakSTAR(S34G,G36R,H43R), SakSTAR(G36R), SakSTAR(H43R), SakSTAR(G36R,K74R), SakSTAR(K35E), SakSTAR(K74Q), SakSTAR(K130T), SakSTAR(V132L), SakSTAR(V132T), SakSTAR(V132N), SakSTAR(V132R), SakSTAR(K130T,K135R), SakSTAR(G36R,K130T,K135R), SakSTAR(K74R,K130T,K135R), SakSTAR(K74Q,K130T,K135R), SakSTAR(G36R,K74R,K130T, K135R), SakSTAR(G36R,K74Q,K130T,K135R), SakSTAR(G36R, H43R,K74R,K130T,K135R), SakSTAR(E65A,K74Q,K130T,K135R), SakSTAR(E65Q,K74Q,K130T,K135R), SakSTAR(K74Q,K86A, K130T,K135R), SakSTAR(E65Q,T71S,K74Q,K130T,K135R), SakSTAR(K74Q,K130A,K135R), SakSTAR(E65Q,K74Q,K130A, K135R), SakSTAR(K74Q,K130E,K135R), SakSTAR(K74Q,K130E, V132R,K135R), SakSTAR(E65Q,K74Q,T90A,K130A,K135R), SakSTAR(E65Q,K74Q,N95A,K130A,K135R), SakSTAR(E65Q,K74Q, E118A,K130A,K135R), SakSTAR(E65Q,K74Q,N95A,E118A,K130A, K135R), SakSTAR(N95A,K130A,K135R), SakSTAR(E65Q,K74Q, K109A,K130,K135R), SakSTAR(E65Q,K74Q,E108A,K109A,

K130T,K135R), SakSTAR(E65Q,K74Q,K121A,K130T,K135R), SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,+137K), SakSTAR(E80A,D82A,K130T,K135R), SakSTAR(K74R,E80A,D82A, K130T,K135R), SakSTAR(K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A,K74R,E80A,D82A,K130T,K135R), SakSTAR(E65D, K74R,E80A,D82A,K130T,K135R), SakSTAR(E65S,K74R,E80A, D82A,K130T,K135R), SakSTAR(S34G,G36R,K74R,K130T,K135R), SakSTAR(E65A,K74R,E80A,D82A,K130T,K135R), SakSTAR(E65N, K74R,E80A,D82A,K130T,K135R), SakSTAR(E65Q,K74R,E80A, D82A,K130T,K135R), SakSTAR(K57A,E58A,E61A,E80A,D82A, K130T,K135R), SakSTAR(E65D,K74Q,E80A,D82A,K130T,K135R), SakSTAR(E65Q,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A, E65D,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K74R,E80A,D82A, S103A,K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K109A, K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K130T, K135R,K136A), SakSTAR(E65Q,K74Q,D82A,S84A,K130T,K135R), SakSTAR(K35A,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A, E65D,K74R,E80A,D82A,K130T,K135R).

Of these SakSTAR(E65D,K74R,E80A,D82A,K130T, K135R) having the code SY19 and SakSTAR(K35A,E65Q,K74R, E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T,K135R) having the code SY161 are especially preferred.

Besides the above described substitution derivatives the invention relates to derivatives having in addition an amino acid substituted with Cys. This type of substitution may result in dimerization and/or increased specific activity and/or reduced clearance and/or increased thrombolytic potency. Reduced plasma clearance is in particular obtained when the derivative is substituted with polyethylene glycol.

Preferred embodiments of such staphylokinase derivatives are those wherein the Cys is chemically modified with polyethylene glycol with molecular weights up to 20 kDa. In particular embodiments selected amino acids in the NH_2-terminal region of 10 amino acids, are substituted with Cys, which is chemically modified with polyethylene glycol with molecular weights up to 20 kDa. These derivatives are characterized by a significantly

reduced plasma clearance and maintained thrombolytic potency upon single intravenous bolus administration at a reduced dose.

More in particular the serine in position 2 or 3 is substituted with a cystein and the cystein is chemically modified with polyethylene glycol having a molecular weight of 5, 10 or 20 kDa. Preferred embodiments of these derivatives are SY161(S3C-MP5), SY161(S3C-P10), SY161(S3C-P20), SY19(S3C-MP5), SY19(S3C-SP5), SY19(S2C-SP5,S3C-SP5), SY19(S3C-P20), SY19(S3C-P10) all as defined in table 20.

The presence of cysteins allows the formation of dimers of two staphylokinase derivatives of the invention.

The invention also relates to a method for producing the derivatives of the invention by preparing a DNA fragment comprising at least the part of the coding sequence of staphylokinase that provides for its biological activity; performing in vitro site-directed mutagenesis on the DNA fragment to replace one or more codons for wild-type amino acids by a codon for another amino acid; cloning the mutated DNA fragment in a suitable vector; transforming or transfecting a suitable host cell with the vector; culturing the host cell under conditions suitable for expressing the DNA fragment; purifying the expressed staphylokinase derivative to homogeneity and derivatizing the variant with polyethylene glycol.

Preferably the DNA fragment is a 453 bp EcoRI-HindIII fragment of the plasmid pMEX602sakB (22, 23), the in vitro site-directed mutagenesis is preferably performed by spliced overlap extension polymerase chain reaction. Such overlap extension PCR is preferably performed with Vent DNA polymerase (New England Biolabs) or Taq polymerase (Boehringer Mannheim) and with available or generated wildtype sakSTAR or sakSTAR variants as template (24).

The invention also relates to pharmaceutical compositions comprising at least one of the staphylokinase derivatives according to the invention together with a suitable excipient, for treatment of arterial thrombosis. Pharmaceutical compositions, containing less immunogenic staphylokinase variants or "pegylated" staphylokinase variants as the active ingredient, for treating arterial thrombosis in human or veterinary practice may take the form of powders or solutions and may be used for intravenous, intraarterial or parenteral administration. Such compositions may be prepared by combining (e.g. mixing, dissolving etc.) the active compound with pharmaceutically acceptable excipients of neutral character (such as aqueous or non-aqueous solvents, stabilizers, emulsifiers, detergents, additives), and further, if necessary with dyes.

Furthermore the invention relates to the use of the staphylokinase derivatives for the treatment of arterial thrombosis, in particular myocardial infarction, and to the use of staphylokinase derivatives for the preparation of a pharmaceutical composition for the treatment of arterial thrombosis, in particular myocardial infarction. In the above and the following the terms "derivatives", "mutants" and "variants" are used interchangeably.

Based on the present invention other variants and improvements will be obvious for the person skilled in the art. Thus random mutagenesis is likely to generate alternative mutants with reduced immunogenicity and possibly increased functional activity, whereas deletions or substitution with other amino acids may yield additional variants with reduced immunogenicity.

The present invention will be demonstrated in more detail in the following examples, that are however not intended to be limiting to the scope of the invention. In the Examples reference is made to the following figures:

Fig 1. Protein sequence of wild-type staphylokinase, SakSTAR. Numbering starts with the NH2-terminal amino acid of mature full length staphylokinase.

Fig 2. Time course of neutralizing activities (left panel) and specific IgG against administered agent (right panel) following intra-arterial infusion of SakSTAR (open circles, n= 9), SakSTAR(K74A) (closed circles, n= 11) or SakSTAR(K74A,E75A, R77A) (open squares, n= 6) in patients with peripheral arterial occlusion. The data represent median values and interquartile ranges, in μg/mL.

Fig 3. Protein sequence of wild-type staphylokinase, SakSTAR with indicated amino acid substitititions.

squares: single amino acid substitutions;
circles: combined (2 to 3) amino acid to Ala substitutions.

Fig. 4. Temperature stability of SakSTAR, (A); SakSTAR(K74Q,E80A,D82A,K130T, K135R) (B); SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R), (C); and SakSTAR(K35A,E65D,K74Q,E80A,D82A, K130T,K135R), (D). (○): 4°C; (●): 20°C; (▽): 37°C; (▼): 56°C; (□): 70°C.

Fig 5. Time course of neutralizing activities (left panel) and specific IgG against administered agent (right panel) following intra-arterial infusion of SakSTAR (circles, n= 6), SakSTAR(K74Q,E80A,D82A,K130T,K135R) (squares, n= 6) or SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) (triangles, n= 6) in patients with peripheral arterial occlusion. The data represent median values and 15-85 percentile ranges, in μg/mL.

EXAMPLES

EXAMPLE 1

Epitope mapping of wild-type staphylokinase

The epitope specificity of a panel of 15 murine MAbs (22) raised against wild-type SakSTAR was determined

by real-time biospecific interaction analysis (BIA) with the BIAcore instrument (Pharmacia, Biosensor AB, Uppsala, Sweden). The MAbs were immobilized on the surface of the Sencor Chip CM5 with the Amine Coupling Kit (Pharmacia Biosensor AB) as recommended by the manufacturer (25). Immobilization was performed from protein solutions at a concentration of 20 μg/mL in 10 mmol/L sodium acetate at pH 5.0 at a flow rate of 5 μL/min during 6 minutes. This resulted in covalent attachment of 5,000 to 10,000 resonance unit (RU) of antibody (corresponding to 0.035 to 0.07 pmol/mm^2). The SakSTAR solutions were passed by continuous flow at 20°C past the sensor surface. At least four concentrations of each analyte (range, 50 nmol/L to 50 mol/L) in 10 mmol/L HEPES, 3.4 mmol/L EDTA, 0.15 mol/L NaCl, and 0.005% Surfactant P20, pH 7.2, were injected at a flow rate of 5 μL/min during 6 minutes in the association phase. Then sample was replaced by buffer, also at a flow rate of 5 μL/min during 6 minutes. After each cycle, the surface of the sensor chip was regenerated by injection of 5 μL of 15 mmol/L HCl. Apparent association (k_{ass}) and apparent dissociation (k_{diss}) rate constants were derived from the sensorgrams as described in detail elsewhere (26), and association equilibrium constants (K_A) calculated as their ratio.

Determination of the equilibrium association constants for the binding of wild-type and variant SakSTAR to insolubilized MAbs (Table 1) yielded apparent association constants of 10^7 to 10^8 (mol/L)$^{-1}$, which are one to two orders of magnitude lower than the apparent association constants previously obtained for the binding of these MAbs to insolubilized wild-type SakSTAR (22). If the MAbs instead of the SakSTAR variants are insolubilized, avidity effects of the bivalent MAbs are avoided. The present values are indeed in better agreement with known association constants of Mabs, and therefore this "reversed" procedure was used throughout the present invention.

In the tables the column indicated with "Variant" states the various staphylokinase derivatives which are identified by listing between brackets the substituted amino acids in single letter symbols followed by their position number in the mature staphylokinase sequence and by the substituting amino acids in single letter symbol; the column "Exp." indicates expression levels in mg/L, and the column "Spec. Act." indicates the specific activity in Home Units as defined in example 2. Indications "17G11", "26A2" etc. refer to monoclonal antibodies binding to the indicated epitopes I, II and III as defined in reference 22. Epitope I is recognized by the antibody cluster 17G11, 26A2, 30A2, 2B12 and 3G10, whereas epitope II is recognized by the antibody cluster 18F12, 14H5, 28H4, 32B2 and 7F10, and epitope III by the antibody cluster 7H11, 25E1, 40C8, 24C4 and 1A10. Human plasma "Pool" indicates a plasma pool from initially 16 and subsequently 10 patients immunized by treatment with SakSTAR, "Subpool B" indicates a plasma pool from three patients that absorbed less than 50% of the induced antibodies with SakSTAR(K35A,E38A,K74A,E75A,R77A) and "Subpool C" indicates a plasma pool from 3 patients that absorbed >90% of the induced antibodies with SakSTAR(K35A,E38A,K74A,E75A,R77A) (22).

In tables 6, 7 and 8 an additional pool of plasma from 40 patients immunized by treatment with SakSTAR (Pool 40) was also used.

EXAMPLE 2

<u>Construction, epitope mapping with murine monoclonal antibodies and absorption with pooled plasma of immunized patients, of "alanine-to-wild-type" reversal variants of "charged-cluster-to-alanine" mutants of staphylokinase</u>

1. <u>Introduction</u>

As stated above, wild-type staphylokinase (SakSTAR variant (9)) contains three non-overlapping immunodominant epitopes, two of which can be eliminated by specific site-directed substitution of clusters of two

(K35A,E38A or E80A,D82A) or three (K74A,E75A,R77A) charged amino acids with Ala (22). The combination mutants SakSTAR(K35A,E38A,K74A,E75A,R77A) in which Lys35, Glu38, Lys74, Glu75 and Arg77, and SakSTAR(K74A,E75A, R77A,E80A,D82A) in which Lys74, Glu75, Arg77, Glu80 and Asp82 were substituted with Ala (previously identified as SakSTAR.M3.8 and SakSTAR.M8.9, respectively (22)), were found to have a reduced reactivity with murine monoclonal antibodies against two of the three immunodominant epitopes and to absorb on average only 2/3 of the neutralizing antibodies elicited in 16 patients by treatment with wild-type SakSTAR (22). These mutants also induced less antibody formation than wild-type SakSTAR in experimental thrombolysis models in rabbits and baboons, and in patients with peripheral arterial occlusion (22). However, their specific activities were reduced to approximately 50% of that of wild-type SakSTAR, which would be of some concern with respect to the clinical use of these compounds.

In an effort to improve the activity and stability without loss of the reduced antibody recognition, the effect of a systematic reversal of one or more of these substituted amino acids to the wild-type residues was studied. Fourteen new mutants were constructed, purified and characterized in terms of specific activity, reactivity with the panel of murine monoclonal antibodies, and absorption of antibodies from plasma of patients treated with wild-type SakSTAR (Table 1). The present example thus focusses on reversal from alanine to the wild-type residue of one or more of the seven amino acids of SakSTAR listed above i.e. K35, E38, K74, E75, R77, E80 and D82.

2. <u>Reagents and Methods</u>

The source of all reagents used in the present study has previously been reported (22). Restriction enzymes were purchased from Pharmacia (Uppsala, Sweden) or Boehringer Mannheim (Mannheim, Germany). T4 DNA

ligase, Klenow Fragment of E. coli DNA polymerase I and alkaline phosphatase were obtained from Boehringer Mannheim. Enzyme reactions were performed using the conditions suggested by the suppliers. Plasmid DNA was isolated using a QIAGEN-purification protocol (provided by Westburg, Leusden, The Netherlands). pMEX.602sakB (i.e. pMEX.SakSTAR) was constructed as described elsewhere (23). SakSTAR, SakSTAR(K35A,E38A), SakSTAR(K74A,E75A,R77A), SakSTAR(E80A,D82A), SakSTAR(K35A,E38A,K74A,E75A,R77A) and SakSTAR(K74A,E75A,R77A,E80A,D82A) were produced and purified as described elsewhere (22). Transformations of E. coli were performed utilizing the calcium phosphate procedure. DNA sequencing was performed using the dideoxy chain termination reaction method and the Automated Laser fluorescent A.L.F.™ (Pharmacia). The chromogenic substrate (S2403) L-Pyroglutamyl-L-phenylalanyl-L-lysine-p-nitroanaline hydrochloride was purchased from Chromogenix (Belgium). ^{125}I-labeled fibrinogen was purchased from Amersham (UK). All other methods used in the present example have been previously described (22,27).

3. Construction of expression plasmids

The plasmids encoding SakSTAR(K35A,E38A,K74A,E75A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(K74A) and SakSTAR(E75A) were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24), using Vent DNA polymerase (New England Biolabs, Leusden, The Netherlands), and available or generated sakSTAR variants as template. Two fragments were amplified by PCR, the first one starting from the 5' end of the staphylokinase gene with primer 5'-CAGGAAACAGAATTCAGGAG-3' to the region to be mutagenized (forward primer), the second one from the same region (backward primer) to the 3' end of the

staphylokinase gene with primer 5'-CAAAACAGCCAAGCTTCATTCATTCAGC-3'. The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a new primerless PCR using Taq polymerase (Boehringer Mannheim). After 7 cycles (1 min at 94°C, 1 min at 70°C), the extended product was reamplified by adding the 5' and 3' end primers (see above) to the PCR reaction and by cycling 25 times (1 min at 94°C, 1 min 55°C, 1 min at 72°C). The final product was purified, digested with EcoRI and HindIII and cloned into the corresponding sites of pMEX602sakB. The plasmid encoding SakSTAR(E38A,K74A,E75A,R77A) was assembled by digestion of pMEX602sakB and pMEX.SakSTAR(K35A,E38A,K74A,E75A,R77A) with BpmI which cuts between the codons for K35 and E38 of SakSTAR, and ligation of the required fragments. The plasmid encoding SakSTAR(K35A,K74A,E75A,R77A) was constructed by digestion of pMEX.SakSTAR(K35A,E38A,K74A,E75A,R77A) and pMEX.SakSTAR(K74A,E75A,R77A) with BpmI and religation of the required fragments. The plasmids encoding SakSTAR(K35A,E38A, E75A,R77A) and SakSTAR(K35A,E38A,K74A,R77A) were constructed by two PCR using pMEX.SakSTAR(K35A,E38A,K74A,E75A,R77A) as template, followed by restriction ligation and recloning into pMEX602sakB.

4. <u>Expression and purification of SakSTAR variants</u>

The SakSTAR variants were expressed and purified, as described below, from transformed <u>E. coli</u> WK6 grown either in LB medium [SakSTAR(E38A,K74A,E75A,R77A), SakSTAR(K74A), SakSTAR(E75A) and SakSTAR(E75A,D82A)], or in terrific broth (TB) (28) medium [SakSTAR(K35A,K74A,E75A,R77A), SakSTAR(K35A,E38A,E75A,R77A), SakSTAR(K35A,E38A,K74A,R77A), SakSTAR(K35A,E38A,E75A), SakSTAR(E38A,E75A, R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E80A), and SakSTAR(D82A)].

For derivatives produced in LB medium, a 20 mL aliquot of an overnight saturated culture was used to inoculate a 2 L volume of LB medium containing 100 g/mL ampicillin. After 3 hours incubation at 37°C, IPTG (200 mol/L) was added to induce expression from the tac promoter. The production phase was allowed to proceed for 4 hours, after which the cells were pelleted by centrifugation at 4,000 rpm for 20 min, resuspended in 1/20 volume (100 mL) of 0.01 mol/L phosphate buffer pH 6.5 and disrupted by sonication at 0°C. Cell debris were removed by centrifugation for 20 min at 20,000 rpm and the supernatant, containing the cytosolic soluble protein fraction, was stored at -20°C until purification.

For the derivatives produced in TB medium, a 4 mL aliquot of an overnight saturated culture in LB medium was used to inoculate a 2 L culture in terrific broth containing 100 μg/mL ampicillin. The culture was grown with vigorous aeration for 20 hours at 30°C. The cells were pelleted by centrifugation, resuspended in 1/10 volume (200 mL) of 0.01 mol/L phosphate buffer pH 6.5 and disrupted by sonication at 0°C. The suspension was then centrifuged for 20 min at 20,000 rpm and the supernatant was stored at -20°C until purification. Cleared cell lysates containing the SakSTAR variants were subjected to chromatography on a 1.6 x 6 cm column of SP-Sephadex, followed by chromatography on a 1.6 x 5 cm column of Q-Sepharose [variants SakSTAR(E38A,K74A,E75A,R77A), SakSTAR(K35A,K74A,E75A, R77A), SakSTAR(K35A,E38A, E75A,R77A), SakSTAR(K35A,E38A,K74A,R77A) and SakSTAR(K35A, E38A,K74A,E75A)] or by chromatography on a 1.6 x 6 cm column of phenyl-Sepharose [variants SakSTAR(E35A,E38A,R77A), SakSTAR(E38A,E75A), SakSTAR-(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(K74A), SakSTAR(E75A), SakSTAR(E80A), SakSTAR(D82A) and SakSTAR(E75A,D82A)]. The SakSTAR containing fractions, localized by SDS-gel electrophoresis, were pooled for further analysis.

5. <u>Physicochemical and biochemical analysis</u>

Protein concentrations were determined according to Bradford (29). The specific activities of SakSTAR solutions were determined with a chromogenic substrate assay carried out in microtiter plates using a mixture of 80 μL SakSTAR solution and 100 μL Glu-plasminogen solution prepared as described elsewhere (30) (final concentration 0.5 μmol/L). After incubation for 30 min at 37°C, generated plasmin was quantitated by addition of 20 μL S2403 (final concentration 1 mmol/L) and measurement of the absorption at 405 nm. The activity was expressed in home units (HU) by comparison with an in-house standard (lot STAN5) which was assigned an activity of 100,000 HU (100 kHU) per mg protein as determined by amino acid composition (7). SDS-PAGE was performed with the Phast System™ (Pharmacia, Uppsala, Sweden) using 10-15% gradient gels and Coomassie Brilliant blue staining. Reduction of the samples was performed by heating at 100°C for 3 min in the presence of 1% SDS and 1% dithiothreitol. The specific activities of the different SakSTAR mutants determined with the chromogenic substrate assay are summarized in Table 1.

6. <u>Binding to murine monoclonal antibodies</u>

In agreement with previous observations (22), SakSTAR(K74A,E75A,R77A) did not react with 4 of the 5 MAbs recognizing epitope I, whereas SakSTAR(K35A,E38A) did not react with 3 of the 5 and SakSTAR(E80A,D82A) not with 4 of the 5 Mabs recognizing epitope III. These reduced reactivities were additive in SakSTAR(K35A,E38A, K74A,E75A,R77A) and in SakSTAR(K74A,E75A,R77A,E80A,D82A). The reduced reactivity of SakSTAR(K74A,E75A, R77A) was fully maintained in SakSTAR(K35A,E38A,K74A,E75A) and in SakSTAR(K35A, E75A,R77A), largely in SakSTAR(K35A,E38A, E75A,R77A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A) and SakSTAR(E75A), but much less in SakSTAR(K35A,E38A, K74A,R77A) and SakSTAR(K74A), indicating that E75 is the main contributor to the binding of the 4 Mabs recognizing

epitope I of SakSTAR. However, surprisingly, binding of epitope I antibodies to SakSTAR(E75A,D82A) was normal in two independent preparations from expression plasmids with confirmed DNA sequences. The reduced reactivity of the 3 MAbs of epitope III with SakSTAR(K35A,E38A) required both K35 and E38, as demonstrated with SakSTAR(E38A,K74A,E75A,R77A) and SakSTAR(K35A,K74A,E75A,R77A), with SakSTAR(E38A,E75A) and SakSTAR(K35A,E75A) and with SakSTAR(E38A,E75A,R77A) and SakSTAR(K35A,E75A,R77A). The reduced reactivity of the 4 MAbs of cluster III with SakSTAR(E80A,D82A) was maintained in SakSTAR(D82A) but not in SakSTAR(E80A).

7. <u>Absorption of antibodies, elicited in patients by treatment with wild-type SakSTAR</u>

Plasma samples from 16 patients with acute myocardial infarction, obtained several weeks after treatment with SakSTAR (4, 31) were used. The staphylokinase-neutralizing activity in these samples was determined as follows. Increasing concentrations of wild-type or variant SakSTAR (50 μL volumes containing 0.2 to 1000 μg/mL) were added to a mixture of 300 μL citrated human plasma and 50 μL buffer or test plasma, immediately followed by addition of 100 μL of a mixture containing thrombin (50 NIH units/mL) and $CaCl_2$ (25 mmol/L). The plasma clot lysis time was measured and plotted against the concentration of SakSTAR moiety. From this curve the concentration of staphylokinase moiety that produced complete clot lysis in 20 min was determined. The neutralizing activity titer was determined as the difference between the test plasma and buffer values and was expressed in μg per mL test plasma. The results of the individual patients have been reported elsewhere (22). For the present invention, three plasma pools were made, one from 10 patients from whom sufficient residual plasma was available, one from three patients that absorbed less than 50% of the antibodies with SakSTAR(K35A,E38A, K74A,E75A,R77A) (Subpool B) and

one from three patients that absorbed >90% of the antibodies with SakSTAR(K35A,E38A,K74A,E75A, R77A) (Subpool C). These plasma pools were diluted (1/30 to 1/200) until their binding to SakSTAR substituted chips in the BIAcore instrument amounted to approximately 2000 RU. From this dilution a calibration curve for antibody binding was constructed using further serial two-fold dilutions. The plasma pools were absorbed for 10 min with 100 nmol/L of the SakSTAR variants, and residual binding to immobilized SakSTAR was determined. Residual binding was expressed in percent of unabsorbed plasma, using the calibration curve.

The results are summarized in Table 1. Whereas wild-type SakSTAR absorbed more than 95% of the binding antibodies from pooled plasma of the 10 patients, incomplete absorption (<60%) was observed with SakSTAR(K74A,E75A,R77A), SakSTAR(K35A,E38A,K74A, E75A,R77A), SakSTAR(E38A,K74A,E75A,R77A), SakSTAR(K35A,K74A,E75A,R77A), SakSTAR(K35A, E38A,K74A,R77A), SakSTAR(K35A,E38A,K74A,E75A), SakSTAR(K74A) and SakSTAR(K74A,E75A,R77A,E80A,D82A) but absorption was nearly complete with SakSTAR(K35A,E38A), SakSTAR(K35A,E38A,E75A,R77A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E75A), SakSTAR(E80A,D82A), SakSTAR(E80A), SakSTAR(D82A) and SakSTAR(E75A,D82A). These results, surprisingly, demonstrate that approximately 40% of the antibodies elicited in patients by treatment with wild-type SakSTAR depend on K74 for their binding (Table 1). Absorption with pooled plasma from 3 patients from which <50% of the antibodies were absorbed with SakSTAR(K35A,E38A,K74A,E75A,R77A) (Subpool B) confirmed the predominant role of K74 for antibody recognition. As expected, absorption with pooled plasma from 3 patients from which >95% of the antibodies were absorbed with SakSTAR(K35A,E38A,K74A,E75A,R77A) (Subpool C) was nearly complete with all variants tested.

EXAMPLE 3

Comparative thrombolytic efficacy and immunogenicity of SakSTAR(K74A,E75A,R77A) and SakSTAR(K74A) versus SakSTAR in patients with peripheral arterial occlusion

1. Purification of SakSTAR(K74A,E75A,R77A) and SakSTAR(K74A) for use in vivo

A 12 to 24 L culture (in 2 L batches) of the variants SakSTAR(K74A,E75A,R77A), or of SakSTAR(K74A) was grown and IPTG-induced in LB medium supplemented with 100 μg/mL ampicillin, pelleted, resuspended, disrupted by sonication and cleared as described above. The compounds were purified by chromatography on a 5 x 20 cm column of SP-Sephadex, a 5 x 10 cm column of Q-Sepharose and/or a 5 x 13 cm column of phenyl-Sepharose using buffer systems described elsewhere (22, 23). The materials were then gel filtered on sterilized Superdex 75 to further reduce their endotoxin content. The SakSTAR variant containing fractions were pooled, the protein concentration was adjusted to 1 mg/mL and the material sterilized by filtration through a 0.22 μm Millipore filter. The methodology used to determine the biological properties of the final material required for use in vivo is described above and elsewhere (22).

2. Materials and Methods

Staphylokinase-neutralizing activity in plasma was determined as described above. Quantitation of antigen-specific IgG and IgM antibodies was performed using enzyme-linked immunosorbent assays in polystyrene microtiter plates essentialy as described previously (22). In the IgG assays, dilution curves of affinospecific anti-SakSTAR IgG antibodies were included on each plate. These antibodies were isolated from plasma obtained from 3 patients, after thrombolytic therapy with wild-type SakSTAR, by chromatography on protein A-Sepharose and on insolubilized SakSTAR, and elution of bound antibodies with 0.1 mol/L glycine-HCl, pH 2.8. The purity of the IgG preparation was confirmed by sodium

dodecylsulfate polyacrylamide gel electrophoresis. In the IgM assays, titers defined as the plasma dilution giving an absorbancy at 492 nm equivalent to that of a 1/640 dilution of pooled plasma were determined and compared with the titer of baseline samples before treatment (median value 1/410, interquartile range 1/120-1/700).

3. Thrombolytic efficacy

Wild-type SakSTAR or the variants SakSTAR(K74A) or SakSTAR(K74A,E75A,R77A) were administered intra-arterially at or in the proximal end of the occlusive thrombus as a bolus of 2 mg followed by an infusion of 1 mg/hr (reduced overnight in some patients to 0.5 mg/hr) in groups of 6 to 12 patients with angiographically documented occlusion of a peripheral artery or bypass graft of less than 120 days duration. Patients were studied after giving informed consent, and the protocol was approved by the Human Studies Committee of the University of Leuven. Inclusion and exclusion criteria, conjunctive antithrombotic treatment (including continuous intravenous heparin) and the study protocol were essentially as previously described (22).

Relevant baseline characteristics of the individual patients are shown in Table 2. The majority of PAO were at the femoropopliteal level. Two iliac stent and 8 graft occlusions were included. Eight patients presented with incapacitating claudication, 5 with chronic ischemic rest pain, 7 with subacute ischemia and 7 with acute ischemia. One patient (POE) who had 2 years previously been treated with SakSTAR was included in the SakSTAR(K74A) group. This patient was not included in the statistical analyses.

Table 2 also summarizes the individual treatment and outcome. Intra-arterial infusion, at a dose of 6.0 to 25 mg and a duration of 4.0 to 23 hrs, induced complete recanalization in 24 patients and partial recanalization in 3. Complementary endovascular procedures (mainly PTA) were performed in 17 patients and

complementary reconstructive vascular surgery following thrombolysis in 3. No patient underwent major amputation. Early recurrence of thrombosis after the end of the angiographic procedure occurred in 4 patients. Bleeding complications were absent or limited to mild to moderate hematoma formation at the angiographic puncture sites except for 5 patients who required transfusion (data not shown). Intracranial or visceral hemorrhage was not observed. Circulating fibrinogen, plasminogen and α_2-antiplasmin levels remained essentially unchanged during infusion of the SakSTAR moieties (data not shown), confirming absolute fibrin specificity of staphylokinase at the dosages used. Significant in vivo fibrin digestion occurred as evidenced by elevation of fibrin fragment D-dimer levels. Intra-arterial heparin therapy prolonged aPTT levels to a variable extent (data not shown).

4. Antibody induction

Antibody-related SakSTAR-, SakSTAR(K74A)- and SakSTAR(K74A,E75A,R77A)-neutralizing activity and anti-SakSTAR, anti-SakSTAR(K74A) and anti-SakSTAR(K74A, E75A,R77A) IgG, were low at baseline and during the first week after the infusion (Figure 2). From the second week on, neutralizing activity levels increased to reach median values at 3 to 4 weeks of 20 μg SakSTAR(K74A) and 2.4 μg SakSTAR(K74A,E75A,R77A) neutralized per mL plasma in the patients treated with SakSTAR(K74A) and SakSTAR(K74A,E75A,R77A), respectively, which is significantly lower than the median value of 93 μg wild-type SakSTAR neutralized per mL in the patients treated with SakSTAR ($p= 0.024$ for differences between the three groups by Kruskal-Wallis analysis and $p= 0.01$ and $p= 0.036$, respectively, for variants vs wild-type by Mann-Whitney rank sum test). The levels of anti-SakSTAR(K74A) and of anti-SakSTAR(K74A,E75A,R77A) IgG increased to median values at 3 to 4 weeks of 270 and 82 μg/mL plasma in patients treated with SakSTAR(K74A) and SakSTAR(K74A,E75A,R77A) respectively, which is

significantly lower than the median value of 1800 μg anti-SakSTAR per mL plasma in the patients treated with SakSTAR ((p= 0.024 for differences between the three groups by Kruskal-Wallis analysis and p= 0.007 and 0.05, respectively, for variants versus wild-type by Mann-Whitney rank sum test).

The titers of anti-SakSTAR(K74A) and of anti-SakSTAR(K74A,E75A,R77A) IgM increased from median baseline values of 1/460 and 1/410 to median values at 1 week of 1/510 and 1/450 in patients treated with SakSTAR(K74A) and SakSTAR(K74A,E75A,R77A), respectively, which was not significantly different from the median values of 1/320 at baseline and 1/640 at week 1 in patients treated with SakSTAR. Corresponding values at 2 weeks were 1/590 and 1/550 in patients given SakSTAR(K74A) and SakSTAR(K74A,E75A,R77A), not significantly different from 1/930 with SakSTAR (data not shown). The antibodies induced by treatment with SakSTAR were completely absorbed by SakSTAR but incompletely by SakSTAR(K74A) and by SakSTAR(K74A,E75A,R77A) confirming the immunogenicity of the K74,E75,R77 epitope and the dominant role of K74 in the binding of antibodies directed against this epitope. The antibodies induced by treatment with SakSTAR(K74A) or SakSTAR(K74A,E75A,R77A) were completely absorbed by SakSTAR, by SakSTAR(K74A) and by SakSTAR(K74A,E75A,R77A), indicating that immunization was not due to neoepitopes generated by substitution of Lys74 with Ala, but to epitopes different from the K74,E75,R77 epitope.

Thus, this example illustrates that staphylokinase variants with reduced antibody induction but intact thrombolytic potency can be generated. The present experience in 26 patients treated with SakSTAR (n= 9), SakSTAR(K74A) (n= 11) and SakSTAR(K74A,E75A,R77A) (n= 6) combined with previous experience in 14 patients with SakSTAR (n= 7) and SakSTAR(K35A, E38A,K74A,E75A,R77A) (n= 7) (31) and in 24 patients with SakSTAR (32), and with subsequent non-randomized

experience in patients with SakSTAR (n= 30) with SakSTAR(K74A) (n= 12) and with SakSTAR(K74A,E75A,R77A) (n= 7) (data not shown), allows an initial estimation of the prevalence of immunization by intra-arterial treatment with SakSTAR or variants with an altered K74,E75,R77 epitope [SakSTAR(K74A), SakSTAR(K74A, E75A,R77A) and SakSTAR(K35A,E38A,K74A,E75A, R77A)]. Neutralizing activity data after 2 to 4 weeks, available in 70 patients with peripheral arterial occlusion given intra-arterial SakSTAR, revealed that 56 patients (80 percent) had levels > 5 μg compound neutralized per mL plasma. Of the patients given SakSTAR(K74A), SakSTAR(K74A,E75A, R77A) or SakSTAR(K74A,E75A,K74A,E75A, R77A), 27 of the 43 (63 percent) had neutralizing activity levels of > 5 μg compound per mL plasma. This difference is statistically significant (p= 0.05 by Fisher's exact test) indicating that the K74,E75,R77 epitope is a major determinant of antibody induction.

EXAMPLE 4

Construction, epitope mapping with murine monoclonal antibodies and absorption with pooled plasma of immunized patients, of alanine-substitution mutants of staphylokinase

1. Introduction

Site-directed mutagenesis was applied to residues other than "charged amino acids" in order to identify i) additional residues belonging to epitopes I and III identified with the panel of murine Mabs and ii) amino acids determining absorption to antiserum from immunized patients. Since functional epitopes generally comprise more than one amino acid residue critical for antibody binding, identification of additional residues in these epitopes could lead to the construction of new combination derivatives displaying a lower antigenic profile, while keeping the specific activity and the temperature stability of wild-type staphylokinase. In this example, the construction and characterization of

SakSTAR variants in which one or at most two amino acids (adjacent or in close vicinity) were substituted with alanine is described. The mutants described under this example are listed in Table 3. These variants were expressed in E. coli, purified and characterized in terms of specific activity, reactivity with the panel of murine monoclonal antibodies, and absorption of antibodies from plasma of patients treated with wild-type SakSTAR.

2. Reagents and Methods

The source of all reagents used in the present study has previously been reported (22), or is specified below. The template vector for mutagenesis, pMEX602sakB (i.e. pMEX.SakSTAR), has been described elsewhere (23). Restriction and modification enzymes were purchased from New England Biolabs (Leusden, The Netherlands), Boehringer Mannheim (Mannheim, Germany) or Pharmacia (Uppsala, Sweden). The enzymatic reactions were performed according to the supplier recommendation. The mutagenic oligonucleotides and primers were obtained from Eurogentec (Seraing, Belgium). Plasmid DNA was isolated using a purification kit from Qiagen (Hilden, Germany) or the BIO 101 RPM kit (Vista, CA), as recommended. Transformation-competent E. coli cells were prepared by the well-known calcium phosphate procedure. Nucleotide sequence determination was performed on double strand plasmid DNA with the dideoxy chain termination method, using the T7 sequencing kit (Pharmacia, Uppsala, Sweden). Polymerase chain reactions (PCR) were performed using Taq polymerase from Boehringer Mannheim (Mannheim, Germany) or Vent polymerase (New England Biolabs, Leusden, The Netherlands). The recombinant DNA methods required to construct the variants described in this example are well established (22, 27).

3. Construction of expression plasmids

The variants SakSTAR(Y17A,F18A), SakSTAR(F104A), SakSTAR(F111A), SakSTAR(Y9A), SakSTAR(Y91A),

SakSTAR(Y92A), SakSTAR(I87A), SakSTAR(I106A) and Sak-STAR(I120A) were constructed with the Chameleon Double-Stranded Site-Directed Mutagenesis kit from Stratagene (La Jolla, USA), using the pMEX.SakSTAR vector as template, and following instructions of the supplier. The mutagenic oligonucleotides (not shown) were used in combination with the selection-primer LY34 5' CAAAACAGCCGAGCTTCATTCATTCAGC, which destroys the unique HindIII site located 3' to the staphylokinase encoding gene in pMEX.SakSTAR and allows to counter-select the non-mutant progeny by HindIII digestion. The deletion of the HindIII site was in most cases correlated with the presence of the desired mutation introduced by the mutagenic oligonucleotide. The variant SakSTAR(I133A), was constructed by performing a polymerase chain reaction on the pMEX.SakSTAR plasmid using the primer 818A located at the 5' end of the sakSTAR gene (5' CAGGAAACAGAATTCAGGAG) and the mutagenic primer LY58 (5' TTCAGCATGCTGCAGTTATTTCTTTTCTGCAACAACCTTGG). The amplified product (30 cycles: 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C) was purified, digested with EcoRI and PstI, and ligated into the corresponding sites of pMEXSakSTAR. The variants SakSTAR(I128A), SakSTAR(L127A) and SakSTAR(N126V) were constructed by performing a polymerase chain reaction using the primer 818A located at the 5' end of the sakSTAR gene and mutagenic primers (not shown). The amplified product (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C) was purified, digested with EcoRI and StyI, and ligated into the corresponding sites of pMEXSakSTAR.

The variant SakSTAR(F125A) was constructed by performing two consecutive PCR reactions (30 cycles: : 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C). In the first reaction, a fragment of pMEX.SakSTAR was amplified with the prim rs 818A and a mutagenic primer. This amplified fragment was then used as template in a second PCR reaction with a mutagenic primer in order to further elongate the fragment downstream of the StyI site present

in the sakSTAR gene (corresponding to amino acids 130-131 of SakSTAR). The resulting product was digested with EcoRI and StyI, and ligated into the corresponding sites of pMEXSakSTAR.

The plasmids encoding all the other variants listed in Table 3 were constructed by direct PCR or by the spliced overlap extension polymerase chain reaction (SOE-PCR)(24) using pMEX.SakSTAR or available plasmids encoding SakSTAR variants as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the staphylokinase gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC). The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of pMEX.SakSTAR. For each construction, the sequence of the variant was confirmed by sequencing the entire SakSTAR coding region.

4. <u>Expression and purification of SakSTAR variants</u>

The SakSTAR variants were expressed and purified, as described below, from transformed <u>E. coli</u> grown in terrific broth (TB) medium (28). A 2 to 4 mL aliquot of an overnight saturated culture in LB medium was used to inoculate a 1 to 2 L culture in terrific broth supplemented with 100 μg/mL ampicillin. The culture was incubated with vigorous aeration and at 30°C. After about 16 hours incubation, IPTG (200 μmol/L) was added to the culture to induce expression from the tac promoter. After 3 hours induction, the cells were pelleted by

centrifugation at 4,000 rpm for 20 min, resuspended in 1/10 volume of 0.01 mol/L phosphate buffer pH 6-6.5 and disrupted by sonication at 0°C. The suspension was centrifuged for 20 min at 20,000 rpm and the supernatant was stored at 4°C or at -20°C until purification. The material was purified essentially as described above (Example 2): cleared cell lysates containing the SakSTAR variants were subjected to chromatography on a 1.6 x 5 cm column of SP-Sephadex, followed by chromatography on a 1.6 x 8 cm column of phenyl-Sepharose. The SakSTAR containing fractions, localized by SDS-gel electrophoresis, were pooled for further analysis.

5. <u>Physicochemical and biochemical analysis</u>

Protein concentrations were determined according to Bradford (29). SDS-PAGE was performed with the Phast SystemTM (Pharmacia, Uppsala, Sweden) using 10-15% gradient gels and Coomassie Brillant blue staining, and the specific activities of SakSTAR solutions were determined with a chromogenic substrate assay carried out in microtiter plates (as described in example 2). The specific activity of the different SakSTAR variants are summarized in Table 3.

6. <u>Reactivity of SakSTAR variants with a panel of murine monoclonal antibodies</u>

The methodology used to determine the reactivity of the SakSTAR variants with a panel of murine monoclonal antibodies was described in example 1 above. The results are summarized in Table 3 (the layout of this Table corresponds to the layout of Table 1, as described in example 1). Apparent association constants at least 10-fold lower than those of wild-type staphylokinase were considered as significant and are indicated in bold type in the table.

In order to obtain a comprehensive inventory of the properties of Ala-substitution variants of the SakSTAR molecule, 67 plasmids encoding variants with

substitution of a single or two adjacent amino acids with Ala were constructed, expressed and purified. Together with the 35 charged residue to Ala-substitution variants previously described (22, and example 2), this analysis covers all residues in SakSTAR except Gly, Ala and Pro, as illustrated in Figure 3. Eight of the variants could not be obtained in purified form, primarily as a result of low expression levels, 11 variants were inactive, 56 had a reduced specific activity, and 27 had a maintained or increased specific activity (≥100 kHU/mg). The yields of purified material from cultures of expressed plasmids were 16 mg/L (median, 10 to 90 percentile range 4 to 41 mg/L). SDS polyacrylamide gel electrophoresis consistently showed one main band with $\underline{M}_r \approx 16{,}000$, usually representing 95% of total protein (not shown).

Substitution of K35, N95, S103 or K135 with Ala yielded variants with specific activities of ≥200 kU/mg. Substitution of W66, Y73 or E75 with Ala reduced the reactivity of the variants with ≥3 antibodies of epitope cluster I, of H43 or V45 with Ala that with 3 antibodies from epitope cluster II and of V32, K35, D82 and K130 with Ala that with ≥3 antibodies of epitope cluster III.

7. <u>Absorption of antibodies, elicited in patients by treatment with SakSTAR</u>

For the present example, the three plasma pools, as described in example 2 were used. The methodology used to evaluate the absorption with wild-type staphylokinase and with SakSTAR variants, of antibodies elicited in patients treated with SakSTAR, is described in detail in example 2. The results are summarized in Table 3. Whereas wild-type SakSTAR and most of the variants analyzed in this example absorbed more than 95% of the binding antibodies from pooled plasma of the 10 patients, incomplete absorption (<60%) was observed with SakSTAR(Y73A), and with SakSTAR(K74A). The predominant role of Lys74 for antibody recognition has

been demonstrated previously (see example 2). The present results indicate that Tyr73 participates to the same major epitope as Lys74, or, alternatively, that substitution at Tyr73 may indirectly induce a structural modification of the "K74-epitope". Absorption with pooled plasma from 3 patients from which >95% of the antibodies were absorbed with SakSTAR(K35A,E38A,K74A,E75A,R77A) (Subpool C, see example 2) was nearly complete with most variants tested.

EXAMPLE 5

Construction, epitope mapping with murine monoclonal antibodies and absorption with pooled plasma of immunized patients, of staphylokinase variants with substitution of S34, G36 and/or H43

The natural variant Sak42D differs from SakSTAR in three amino acids and corresponds to SakSTAR(S34G, G36R,H43R). Sak42D is characterized by reduced reactivity with some murine antibodies of epitope clusters II and III and a slightly reduced absorption of antibodies from plasma of patients treated with SakSTAR (Table 4). Mutagenesis of these residues in SakSTAR revealed that the reduced reactivity with epitope cluster III and with immunized patient plasma could be ascribed to the G36R substitution, the H43R substitution mediated the reduced reactivity with epitope cluster II but had no effect on the reactivity with immunized patient plasma, whereas the S34A substitution had no effect. The G36R substitution could be combined with the K74R but not with the K74A substitution, without significant reduction of the specific activity (Table 4).

EXAMPLE 6

Construction, epitope mapping with murine monoclonal antibodies and absorption with pooled plasma of immunized patients, of staphylokinase variants with substitution of K35, E65, Y73, K74, E80+D82, N95, K130, V132 and/or K135

Based on the results of the alanine-substitution analysis in example 4, K35, N95 and K135 were selected for further analysis because SakSTAR(K35A), SakSTAR(N95A) and SakSTAR(K135A) had a two-fold increased specific activity, Y73 and K74 because SakSTAR(Y73A) and SakSTAR(K74A) had a markedly reduced reactivity with antibodies from epitope cluster I and diminished absorption of antibodies from plasma of patients immunized by treatment with SakSTAR, and K35, E80+D82, K130 and V132 because SakSTAR(K35A), SakSTAR(E80A,D82A), SakSTAR(K130A) and SakSTAR(V132A) had a reduced reactivity with antibodies from epitope cluster III.

In an effort to maximize the activity/antigenicity ratio, these amino acids were substituted with other amino acids than Ala. As summarized in Table 5, substitution of K35 with A, E or Q revealed that SakSTAR(K35A) had the most interesting properties, substitution of Y73 with F, H, L, S or W did not rescue the marked reduction in specific activity, and K74 confirmed its key role in binding of antibodies from immunized patient plasma, the best specific activity/antigenicity ratios being obtained with SakSTAR(K74Q) and SakSTAR(K74R). SakSTAR(E80A,D82A) was preferred over the single residue variants SakSTAR(E80A) or SakSTAR(D82A) because of its somewhat lower reactivity with immunized patient plasma. SakSTAR(N95A) could not be further improved by substitution of N95 with E, G, K or R and it was unable to confer its increased specific activity to variants containing K74A or K135R. Finally SakSTAR(K130A) was outperformed in terms of specific activity by SakSTAR(K130T) and SakSTAR(V132A) by SakSTAR(V132R).

EXAMPLE 7

Construction, epitope mapping with murine monoclonal antibodies and absorption with pooled plasma of immunized patients of combination variants of SakSTAR(K130T,K135R) and SakSTAR(E80A,D82A,K130T,K135R) with K35A,G36R,E65X,K74X and selected other amino acids

In the present and the following examples an additional plasma pool was made from 40 patients obtained several weeks after treatment with SakSTAR (Pool 40). The original pool from 10 patients is further identified as Pool 10. The absorption of staphylokinase-specific antibodies was quantified as described above and elsewhere (22).

The SakSTAR(K130T,K135R) variant was taken as a template for additive mutagenesis because of its high specific activity with a moderate reduction of binding to antibodies of epitope cluster III and absorption of antibodies from immunized patient plasma (Table 6). Addition of G36R, K74R, or K74Q or both to the template did not markedly reduce the specific activity, reduced the reactivity with monoclonal antibodies against epitope cluster III (G36R substitution) and decreased the absorption of antibodies from immunized patient plasma (K74R or K74Q substitution). Combination of E65A or E65Q with K74Q in the SakSTAR(K130T,K135R) template reduced the absorption of antibodies from Pool 10 and Pool 40 to around 50 and 60 percent respectively, without markedly reducing the specific activity. Addition substitution of selected amino acids in the SakSTAR(E65Q,K74Q,K130T, K135R) template did not further reduce the antibody absorption from Pool 10 or Pool 40. Surprisingly, the substitution of K136 with A and the addition of K in position 137 resulted in a marked increase in specific activity, as measured in the chromogenic substrate assay.

Combination of the SakSTAR(E80A,D82A) and Sak-STAR(K130T,K135R) templates, did not affect the specific activity and had a reduced reactivity with epitope cluster III antibodies (Table 7). Therefore the Sak-

STAR(E80A,D82A,K130T,K135R) template was selected for further mutagenesis. Addition of K74R and even more of K74Q drastically reduced the reactivity with immunized patient plasma. Finally, addition of E65D or of K35A or E65S to the SakSTAR(K74R,E80A,D82A,K130T,K135R) or Sak-STAR(K74Q,E80A,D82A,K130T, K135R) templates yielded variants with intact specific activity which only bound ≤45 of the antibodies of pooled immunized patient plasma and less than 15 percent of the subpool reacting for more than 50 percent with the K74,E75,R77 epitope.

EXAMPLE 8

Characterization of selected variants of staphylokinase with intact specific activity and less than 50% adsorption of pooled SakSTAR specific human antibodies elicited in patients by treatment with wild-type SakSTAR

1. Introduction

Twenty three of the variants constructed and characterized in the above examples combined the properties of a residual specific activity of ≥100 kHU/mg and ≤50 percent absorption with the pool of antisera obtained from 10 patients treated with wild-type SakSTAR. The results are summarized in Table 8. Results obtained with Subpool B and Subpool C and with the pool of 40 patients treated with wild-type SakSTAR are included. SakSTAR(K74Q,E80A,D82A,K130T, K135R), SakSTAR(E65D,K74R, E80A,D82A,K130T,K135R), SakSTAR(K35A,E65D,K74Q, E80A, D82A,K130T,K135R) and SakSTAR(E65Q,K74Q,N95A,E118A, K130A,K135R,K136A,∇137K) were selected for further characterization.

2. Fibrinolytic properties of SakSTAR variants in human plasma in vitro

The fibrinolytic and fibrinogenolytic properties of the SakSTAR variants were determined as previously described. Dose- and time-dependent lysis of ^{125}I-fibrin labeled human plasma clots submerged in human plasma was obtained with the selected variants (Table 9).

Spontaneous clot lysis during the experimental period was ≤5% (not shown). Equi-effective concentrations of test compound (causing 50% clot lysis in 2 hrs; C_{50}), determined graphically from plots of clot lysis at 2 hrs versus the concentration of plasminogen activator (not shown), ranged from 0.11 ± 0.01 to 0.24 ± 0.04 g/mL at which the residual fibrinogen levels ranges between 92 ± 30 and 97 ± 30 percent of baseline (Table 9). The concentrations of compound causing 50% fibrinogen degradation in 2 hrs in human plasma in the absence of fibrin were determined graphically from dose-response curves (not shown). These values (mean ± SD of 3 independent experiments) ranged from 14 ± 3.2 to 29 ± 3.1 μg/mL (Table 9). Surprisingly the very high specific activity of SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,∇137K) in the chromogenic assay was not associated with an increased thrombolytic potency in a plasma milieu.

3. <u>Temperature stability of selected SakSTAR variants</u>

The temperature stability of preparations of SakSTAR(K74Q,E80A,D82A,K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) and SakSTAR(K35A,E65D,K74Q,E80A,D82A,K130T,K135R), dissolved to a concentration of 1.0 mg/mL in 0.15 mol/L NaCl, 0.01 mol/L phosphate buffer, pH 7.5 at various temperatures is illustrated in Fig. 4. At temperatures up to 37°C, all compounds remained fully active for up at least three days. At 56°C and 70°C the three variants were however less stable than wild-type SakSTAR.

4. <u>Pharmacokinetic properties of SakSTAR variants following bolus injection in hamsters</u>

The pharmacokinetic parameters of the disposition of SakSTAR variants from blood were evaluated in groups of 4 hamsters following intravenous bolus injection of 100 μg/kg SakSTAR variant. SakSTAR-related antigen was assayed using the ELISA described elsewhere.

The ELISA was calibrated against each of the SakSTAR variants to be quantitated. Pharmacokinetic parameters included: initial half-life (in min), $t1/2\alpha= \ln2/\alpha$; terminal half-life (in min), $t1/2\beta=\ln2/\beta$; volume of the central (plasma) compartment (in mL), V_c= dose/(A+B); area under the curve (in $\mu g.min.mL^{-1}$), AUC= $A/\alpha + B/\beta$; and plasma clearance (in $mL.min^{-1}$), Clp= dose/AUC (33).

The disposition rate of staphylokinase-related antigen from blood following bolus injection of 100 μg/kg of the selected SakSTAR variants in groups of 4 hamsters could adequately be described by a sum of two exponential terms by graphical curve peeling (results not shown), from which the pharmacokinetic parameters summarized in Table 10 were derived. The pharmacokinetic parameters of the mutants were not markedly different from those of wild type SakSTAR. Initial plasma half-lives ($t1/2(\alpha)$) ranged between 2.0 and 3.2 min and plasma clearances (Clp) between 1.6 and 4.1 mL/min.

EXAMPLE 9

<u>Comparative thrombolytic efficacy and immunogenicity of SakSTAR(K74Q,E80A,D82A, K130T,K135R) and SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) versus SakSTAR in patients with peripheral arterial occlusion</u>

1. <u>Purification for use in vivo</u>

Eighteen liter cultures (in 2 L batches) of the variants SakSTAR(K74Q,E80A,D82A,K130T, K135R) and SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) were grown for 20 hours in terrific broth medium (28), supplemented with 100 μg/mL ampicillin and induced with IPTG during the last 3 hours. The cells were pelleted, resuspended in 1/10 volume of 0.01 mol/L phosphate buffer, pH 6.0, disrupted by sonication and cleared by centrifugation. The compounds were purified by chromatography on a 10 x 7 cm column of SP-Sepharose, equilibrated with 0.01 mol/L phosphate buffer, pH 6.0 and eluted with a 1 mol/L NaCl gradient (3 column volumes). The fractions containing SakSTAR variant were pooled, solid NaCl was added to a

concentration of 2.5 mol/L and the material was chromatographed on a 10 x 20 cm column of phenyl-Sepharose followed by stepwise elution with 0.01 mol/L phosphate buffer, pH 6.0. The materials were desalted on a 10 x 45 cm column of Sephadex G25, concentrated by application on a 5 x 10 cm column of SP-Sepharose with stepwise elution with 1.0 mol/L NaCl and finally gel filtered on a 6 x 60 cm column of Superdex 75 equilibrated with 0.15 m NaCl, 0.01 mol/L phosphate buffer, pH 7.5 to further reduce their endotoxin content. The SakSTAR variant containing fractions were pooled, the protein concentration was adjusted to 1 mg/mL and the material sterilized by filtration through a 0.22 m Millipore filter. The methodology used to determine specific activity, endotoxin contamination, bacterial sterility and toxicity in mice is described above and elsewhere (22). The purity of the preparation was evaluated by SDS gel electrophoresis on 10% gels to which 40 g of compound was applied.

Out of culture volumes of 18 liters of SakSTAR variant, 840 mg of SakSTAR(K74Q,E80A, D82A,K130T,K135R) with a specific activity of 140 kHU/mg and 800 mg Sak-STAR(E65D, K74R,E80A,D82A,K130T,K135R) with a specific activity of 150 were purified. The endotoxin content was <0.1 and 0.26 IU/mg. Gel filtration on HPLC revealed a single main symmetrical peak in the chromatographic range of the column, representing >98% of the eluted material (total area under the curve) (not shown). SDS gel electrophoresis of 40 g samples revealed single main components (not shown). Preparations sterilized by filtration proved to be sterile on 3 day testing as described elsewhere (22). Intravenous bolus injection of SakSTAR variants in groups of 5 mice (3 mg/kg body weight), did not provoke any acute reaction, nor reduced weight gain within 8 days, in comparison with mice given an equal amount of saline (not shown).

2. <u>Thrombolytic efficacy</u>

Wild-type SakSTAR or the variants SakSTAR(K74Q, E80A,D82A,K130T,K135R) or SakSTAR(E65D,K74R,E80A,D82A, K130T,K135R) were administered intra-arterially at or in the proximal end of the occlusive thrombus as a bolus of 2 mg followed by an infusion of 1 mg/hr (reduced overnight in some patients to 0.5 mg/hr) in groups of 15, 6 and 6 patients respectively with angiographically documented occlusion of a peripheral artery or bypass graft of less than 30 days duration. Patients were studied after giving informed consent, and the protocol was approved by the Human Studies Committee of the University of Leuven. Inclusion and exclusion criteria, conjunctive antithrombotic treatment (including continuous intravenous heparin) and the study protocol were essentially as previously described (22).

Relevant baseline characteristics of the individual patients and results of treatment and outcome are shown in Table 11. Intra-arterial infusion, at a dose of 3.5 to 27 mg and a duration of 2 to 44 hrs, induced complete recanalization in 22 patients and partial recanalization in 5. Complementary endovascular procedures (mainly PTA) were performed in 13 patients and complementary reconstructive vascular surgery following thrombolysis in 5. One patient underwent major amputation. Bleeding complications were usually absent or limited to mild to moderate hematoma formation at the angiographic puncture sites (data not shown). One patient, given wild-type SakSTAR suffered a non-fatal intracranial bleeding, one (BUE) a retroperitoneal hematoma and two (MAN and STRO) a gastro-intestinal bleeding.

Circulating fibrinogen, plasminogen and α_2-antiplasmin levels remained unchanged during infusion of the SakSTAR moieties (data not shown), reflecting absolute fibrin specificity of these agents at the dosages used (data not shown). Significant <u>in vivo</u> fibrin digestion occurred as evidenced by elevation of fibrin

fragment D-dimer levels. Intra-arterial heparin therapy prolonged aPTT levels to a variable extent (data not shown).

3. Antibody induction

Staphylokinase-neutralizing activity in plasma and antigen-specific IgG antibodies were quantitated essentialy as described above and elsewhere (22). Antibody-related SakSTAR-, SakSTAR(K74Q,E80A,D82A, K130T,K135R)- and SakSTAR(E65D,K74R,E80A,D82A, K130T,K135R)-neutralizing activity and anti-SakSTAR, anti-SakSTAR(K74Q,E80A,D82A,K130T,K135R) and anti-SakSTAR(E65D,K74R,E80A, D82A,K130T,K135R) IgG, were low at baseline and during the first week after the infusion (Figure 5). From the second week on, neutralizing activity levels increased to reach median values at 3 to 4 weeks of 9 μg SakSTAR(K74Q,E80A,D82A, K130T,K135R) and 0.5 μg SakSTAR(E65D,K74R,E80A,D82A, K130T,K135R) neutralized per mL plasma in the patients treated with the corresponding moieties, respectively, as compared to median value of 24 μg wild-type SakSTAR neutralized per mL in the 15 patients treated with SakSTAR. The levels of anti-SakSTAR(K74Q,E80A,D82A, K130T,K135R) and of anti-SakSTAR(E65D,K74R,E80A, D82A,K130T,K135R) IgG increased to median values at 3 to 4 weeks of 420 and 30 μg/mL plasma in patients treated with the corresponding moieties, respectively, as compared to a median value of 590 μg anti-SakSTAR per mL plasma in the patients treated with SakSTAR (Figure 5). The prevalence of immunization, defined as neutralizing activities in plasma after 2 to 4 weeks exceeding 5 g/ml was 3 of 6 patients (50 percent) with SakSTAR(K74Q,E80A, D82A,K130T,K135R), 1 of 6 patients (17 percent) with SakSTAR(E65D,K74R,E80A,D82A, K130T,K135R), as compared to 56 of 70 patients (80 percent) with SakSTAR. This difference is statistically highly significant ($p= 0.01$ by 2 x 3 Chi square analysis).

The antibodies induced by treatment with SakSTAR were completely absorbed by SakSTAR but incompletely by SakSTAR(K74Q,E80A,D82A,K130T,K135R) and by SakSTAR(E65D,K74R, E80A,D82A,K130T,K135R) (Table 12). Antibodies induced by treatment with Sak-STAR(K74Q,E80A,D82A,K130T,K135R), detectable in 4 of the 6 patients, were completely (≥90 percent) absorbed by SakSTAR, by SakSTAR(K74Q,E80A,D82A,K130T, K135R) and by SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R), indicating that immunization was not due to neoepitopes generated by substitution of wild-type amino acids. Antibodies induced by treatment with SakSTAR(E65D,K74R,E80A,D82A, K130T,K135R) detectable in one patient (URB) were completely absorbed with SakSTAR(K74Q,E80A,D82A, K130T,K135R) and with SakSTAR(E65D,K74Q,E80A,D82A, K130T,K135R) but incompletely (85%) with wild-type SakSTAR, suggesting that a small fraction of the induced antibodies might be directed against a neoepitope in the variant used for infusion.

EXAMPLE 10

<u>Construction and absorption with pooled plasma of immunized patients of combination variants of SakSTAR(E65Q,K74Q,K130T,K135R) and other selected amino acids</u>

1. <u>Introduction</u>

In a final round of additive substitution mutagenesis, the SakSTAR(E65Q,K74Q,K130T, K135R) variant was taken as a template because it displayed a high specific activity with a significant reduction of absorption (to 65 percent) of antibodies from pooled immunized patient plasma (Pool 40). The intermediate variants which were relevant for the composition of the finally selected variants are summarized in Table 13. Addition of K35A, D82A and S84A, of T90A,E99D and T101S or of E108A and K109A reduced the antibody absorption to around 50 percent, whereas the combined addition of D82A,S84A and E108A, K109A reduced it to 41 percent.

Substitution of K136A combined with the addition of a Lys at the COOH terminus (-137K) increased the specific activity in a purified system but not in a plasma milieu nor in a hamster pulmonary embolism model (not shown), and further reduced the absorption of antibodies from pooled patient plasma to 30 percent. Finally, addition of the K35A, and T90A,E99D,T101S substitutions to this template yielded a mutant with intact thrombolytic potency which only bound 24 percent of the antibodies of pooled immunized patient plasma.

Based on this analysis, SakSTAR(E65Q,K74Q,D82A, S84A,E108A,K109A,K130T,K135R, K136A,∇137K), (SY118), and SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S, E108A,K109A,K130T,K135R,K136A,∇137K), (SY141), were selected for further characterization. In addition, SakSTAR(K35A,E65Q,K74R,D82A,S84A,T90A,E99D,T101S, E108A,K109A,K130T,K135R,K136A,∇137K), (SY145) with a Lys in position 74, was constructed and evaluated.

2. Pharmacokinetic properties of SakSTAR variants following bolus injection in hamsters

The disposition rate of staphylokinase-related antigen from blood following bolus injection of 100 μg/kg of the selected SakSTAR variants in groups of 4 hamsters could adequately be described by a sum of two exponential terms by graphical curve peeling (results not shown). The pharmacokinetic parameters of the mutants were derived from these plasma disappearance curves not markedly different from those of wild type SakSTAR (results very similar to those of table 10, data not shown).

EXAMPLE 11

Characterization of selected variants derived from SakSTAR(E65Q,K74Q,K130T,K135R)

1. Fibrinolytic properties of selected SakSTAR variants towards human plasma in vitro

Dose- and time-dependent lysis of ^{125}I-fibrin labeled human plasma clots submerged in human plasma was

obtained with the three selected variants (Table 14). Spontaneous clot lysis during the experimental period was ≤5% (not shown). Equi-effective concentrations of test compound (causing 50% clot lysis in 2 hrs; C_{50}), determined graphically from plots of clot lysis at 2 hrs versus the concentration of plasminogen activator (not shown), ranged from 0.15 ± 0.02 to 0.19 ± 0.01 μg/ml at which no significant fibrinogen degradation occurred. The concentrations of compound causing 50% fibrinogen degradation in 2 hrs in human plasma in the absence of fibrin were determined graphically from dose-response curves (not shown). These values (mean ± SD of 3 independent experiments) ranged from 7.0 ± 0.6 to 24 ± 3.6 μg/ml (Table 14).

2. <u>Temperature stability of selected SakSTAR variants</u>

The temperature stability of preparations of SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R, K136A,∇137K), SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A, E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K), and SakSTAR(K35A,E65Q, K74R,D82A,S84A,T90A,E99D,T101S,E108A, K109A,K130T,K135R,K136A,∇137K) dissolved to a concentration of 1.0 mg/ml in 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.5 at various temperatures. At temperatures up to 37°C, all compounds remained fully active for up to at least three days. At 56°C and 70°C the variants were generally less stable than wild type SakSTAR (results very similar to those of Figure 4, data not shown).

EXAMPLE 12

Comparative thrombolytic efficacy and immunogenicity of SakSTAR(E65Q,K74Q, D82A,S84A,E108A,K109A,K130T,K135R, K136A,∇137K), (SY118), SakSTAR(K35A, E65Q,K74Q,D82A,S84A, T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A, V∇137K), (SY141), and SakSTAR(K35A,E65Q,K74R,D82A,S84A,T90A, E99D,T101S, E108A,K109A,K130T,K135R,K136A,∇137K), (SY145), in patients with peripheral arterial occlusion

1. Large scale purification and conditioning of SakSTAR variants for use in vivo

Material was purified to homogeneity out of culture volumes of 18 liters. The endotoxin content was below 2 IU/mg. Gel filtration on HPLC revealed a single main symmetrical peak in the chromatographic range of the column, representing >98% of the eluted material (total area under the curve) (not shown). SDS gel electrophoresis of 30 μg samples revealed single main components. Preparations sterilized by filtration proved to be sterile on 3 day testing. Intravenous bolus injection of SakSTAR variants in groups of 5 mice (3 mg/kg body weight), did not provoke any acute reaction, nor reduced weight gain within 8 days, in comparison with mice given an equal amount of saline (not shown).

Groups of 6 patients with angiographically documented peripheral arterial occlusion (PAO) were studied. Relevant baseline characteristics of the individual patients are shown in Table 15. Table 16 summarizes the individual treatment and outcome. Intra-arterial infusion, at a dose of 6 to 24 mg and a duration of 4 to 29 hrs, induced complete recanalization in most patients. Circulating fibrinogen, plasminogen and α_2-antiplasmin levels remained essentially unchanged during infusion of the SakSTAR variants (data not shown), reflecting absolute fibrin specificity of these agents at the dosages used. Antibody-related SakSTAR(E65Q,K74Q, D82A,S84A,E108A,K109A,K130T,K135R,K136A,∇137K)-, Sak-STAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,

K109A, K130T,K135R,K136A,∇137K)- and SakSTAR(K35A,E65Q, K74R,D82A,S84A,T90A,E99D, T101S,E108A,K109A,K130T,K135R, K136A,∇137K)-neutralizing activity, were low at baseline and during the first week after the infusion (Table 17). From the second week on neutralizing activity levels increased to reach median values at 3 to 4 weeks of 19 μg SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R, K136A,∇137K), (SY118), 0.7 μg SakSTAR(K35A,E65Q,K74Q, D82A, S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R, K136A,∇137K), (SY141), and 4.3 μg SakSTAR(K35A,E65Q,K74R, D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R, K136A,∇137K), (SY145), neutralized per ml plasma in the patients treated with the respective compounds, which for SY141 and SY145, but not for SY118 is lower than the median value of 12 μg wild type SakSTAR neutralized per ml in 69 patients treated with wild type SakSTAR.

Overt immunization (neutralizing activity at 3 to 4 weeks of 5 g compound per ml plasma) was observed in 56 of 70 patients treated with SakSTAR, in 5 of the 6 patients exposed to SakSTAR(E65Q,K74Q,D82A,S84A,E108A, K109A,K130T,K135R,K136A,∇137K), (SY118), only in 2 of the 6 patients given SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A, E99D, T101S,E108A,K109A,K130T,K135R,K136A,∇137K), (SY141), and in 1 of the 3 patients given SakSTAR(K35A, E65Q,K74R,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T, K135R,K136A,∇137K), (SY145).

The results with respect to immunogenicity of the main variants studied in patients are summarized in Table 18. Clearly, variants SakSTAR(E65D,K74R,E80A,D82A, K130T,K135R) and SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A, E99D,T101S,E108A,K109A,K130T, K135R,K136A,∇137K) have a significantly reduced immunogenicity when compared to the wild type protein.

EXAMPLE 13

Construction, purification and characterization of cysteine-substitution mutants of staphylokinase

1. Introduction

Site-directed mutagenesis was applied to substitute exposed amino acids with single cysteine residues in order to construct i) homodimeric forms of staphylokinase, upon formation of an intermolecular disulfide bridge, and ii) polyethylene glycol-conjugated molecules (PEG-derivatives). The aim of this example was twofold: first, the clearance can be reduced by increasing the size of the injected molecule (via dimerization or conjugation with large molecule such as PEG) and second, PEG-derivatives have also been shown to induce a reduced immunoreactivity in animal models (for review, see ref. 34). In both cases, a prolonged half-life *in vivo* could help to reduce the pharmacological dose of staphylokinase in patients. This reduction could be accompanied with a reduced immunogenic reaction against the thrombolytic agent, thus enhancing its pharmacological activity as a thrombolytic agent.

In this example, the construction and characterization of two SakSTAR variants in which one single amino acid was substituted with cysteine is described. The mutants described under this example are listed in Table 19. These variants were expressed in *E. coli*, purified and characterized in terms of specific activity, fibrinolytic properties in human plasma *in vitro* and pharmacokinetic properties following bolus injection in hamsters.

2. Reagents and Methods

The source of all reagents used in the present study has previously been reported (22), or is specified below. The template vector for mutagenesis, pMEX602sakB (i.e. pMEX.SakSTAR), has been described elsewhere (23). Restriction and modification enzymes were purchased from New England Biolabs (Leusden, The Netherlands),

Boehringer Mannheim (Mannheim, Germany) or Pharmacia (Uppsala, Sweden). The enzymatic reactions were performed according to the supplier recommendation. The mutagenic oligonucleotides and primers were obtained from Eurogentec (Seraing, Belgium). Plasmid DNA was isolated using a purification kit from Qiagen (Hilden, Germany), as recommended. Transformation-competent E. coli cells were prepared by the well-known calcium phosphate procedure. Nucleotide sequence determination was performed on double strand plasmid DNA with the dideoxy chain termination method, using the T7 sequencing kit (Pharmacia, Uppsala, Sweden). Polymerase chain reactions (PCR) were performed using Taq polymerase from Boehringer Mannheim (Mannheim, Germany). The recombinant DNA methods required to construct the variants described in this example are well established (22, 27).

3. Construction of expression plasmids

The variants SakSTAR(K102C) and SakSTAR(K109C), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using pMEX.SakSTAR encoding SakSTAR as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the staphylokinase gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATT-CATTCAGC). The forward and backward primers shared an overlap of around 24 bp (for the construction of K102C: TAT GAT AAG AAT TGC AAA AAA GAA GAA (backward) and TTC TTC TTT TTT GCA ATT CTT ATC ATA (forward), for the construction of K109C: AAA AAG AAG AAA CGT GCT CTT TCC CTA (backward) and TAG GGA AAG AGC ACG TTT CTT CTT TTT (forward)). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product

from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of pMEX.SakSTAR. For each construction, the sequence of the variant was confirmed by sequencing the entire coding region.

4. Expression and purification of SakSTAR variants

The SakSTAR variants were expressed and purified, as described below, from transformed E. coli grown in terrific broth (TB) medium (28). A 2 to 4 mL aliquot of an overnight saturated culture in LB medium was used to inoculate a 1 to 2 L culture in terrific broth supplemented with 100 μg/mL ampicillin. The culture was incubated with vigorous aeration and at 30°C. After about 16 hours incubation, IPTG (200 μmol/L) was added to the culture to induce expression from the tac promoter. After 3 hours induction, the cells were pelleted by centrifugation at 4,000 rpm for 20 min, resuspended in 1/10 volume of 0.01 mol/L phosphate buffer pH 6-6.5 and disrupted by sonication at 0°C. The suspension was centrifuged for 20 min at 20,000 rpm and the supernatant was stored at 4°C or at -20°C until purification. The material was purified essentially as described above (Example 2): cleared cell lysates containing the SakSTAR variants were subjected to chromatography on a 1.6 x 5 cm column of SP-Sephadex, followed by chromatography on a 1.6 x 8 cm column of phenyl-Sepharose. The SakSTAR containing fractions, localized by SDS-gel electrophoresis, were pooled for further analysis.

5. Biochemical analysis

Protein concentrations were determined according to Bradford (29). SDS-PAGE was performed with the Phast System™ (Pharmacia, Uppsala, Sweden) using 10-15% gradient gels and Coomassie Brillant blue staining, and the specific activities of SakSTAR solutions were determined with a chromogenic substrate assay carried out in microtiter plates (as described in

example 2). The specific activity of the different SakSTAR variants are summarized in Table 19.

Mutant SakSTAR(K102C) was essentially monomeric as visualized by SDS-PAGE and Coomassie Brillant blue staining. Its specific activity was comparable to that of wild-type staphylokinase. In contrast, SakSTAR(K109C) showed a propensity to form dimers (> 60%). This resulted in a markedly increased specific activity in the plasminogen-coupled chromogenic substrate assay (see Table 19). Upon reduction with dithiothreitol (DTT) (20-fold molar excess during 1.5 hour at 37°C) and alkylation with iodoacetamide (100-fold molar excess during 1 hour at 37°C), the K109C dimer is converted into a stable monomer and its resulting specific activity is within the expected range towards wild-type staphylokinase (Table 19). This result confirms that formation of homodimers is the unique determinant for this large increase in specific activity. Dimeric SakSTAR(K109C) was separated from monomeric SakSTAR(K109C) by chromatography on Source S (Pharmacia) (5 x 50mm). Loading buffer was 10 mM phosphate, pH 6.0 and dimeric SakSTAR(K109C) was eluted by a salt gradient (up to 1 M) in the same buffer. The dimeric SakSTAR(K109C) (>95% pure) containing fractions, localized by SDS-gel electrophoresis, were pooled for further analysis.

6. <u>Chemical crosslinking of cysteine mutants of SakSTAR with polyethylene glycol</u>

The thiol group of the cysteine mutant SakSTAR(K102C) was targeted for coupling with an activated polyethylene glycol, OPSS-PEG (Shearwater Polymers Europe, Enschede, The Netherlands). OPSS-PEG is a 5 kDa PEG molecule carrying a single activated thiol group at one end that react specifically at slightly alkaline pH with free thiols. Modification of SakSTAR(K102C) was achieved by incubating the molecule (100 µM) with a three-fold excess of SS-PEG in a 5 mM

phosphate, pH 7.9 solution at room temperature. The extent of the reaction was monitored by following the release of 2-thiopyridone from OPSS-PEG at 412 nm. After reaction (about 15 min), the excess of OPSS-PEG was removed by purifying the derivatized SakSTAR(K102C-PEG) on a 1.6 x 5 cm column of SP-Sephadex as described above (see Example 2). The SakSTAR(K102C-PEG) containing fractions, localized by optical density at 280 nm, were pooled for further analysis. SDS-PAGE analysis and Coomassie blue staining confirmed that PEG crosslinking on SakSTAR(K102C) was quantitative. As shown in Table 19, the specific activity of the PEG-derivative was only marginally affected when compared to that of wild-type staphylokinase.

7. <u>Fibrinolytic properties of SakSTAR variants in human plasma in vitro</u>

The fibrinolytic and fibrinogenolytic properties of SakSTAR variants were determined as previously described. Dose- and time-dependent lysis of ^{125}I-fibrin labeled human plasma clots submerged in human plasma was obtained with four molecules: SakSTAR(K109C) as dimer and as monomer (after reduction and alkylation with iodoacetamide), the monomeric SakSTAR(K102C) and the PEG-derivatized SakSTAR(K102C). Spontaneous clot lysis during the experimental period was ≤5% (not shown). Equi-effective concentrations of test compound (causing 50% clot lysis in 2 hrs; C_{50}), determined graphically from plots of clot lysis at 2 hrs versus the concentration of plasminogen activator (not shown), were comparable to that of SakSTAR, for monomeric SakSTAR(K109C) and SakSTAR(K102C) (Table 19). However, it was observed that the C_{50} for clot lysis by dimeric SakSTAR(K109C) was only 0.12 μg/ml, which is approximately three-fold lower than for wild-type staphylokinase. In contrast, a C_{50} of 0.60 μg/ml was measured for SakSTAR(K102C-PEG), which is only two-fold higher than for wild-type staphylokinase. Thus,

dimerization of SakSTAR via disulfide bridges or increasing the size of the molecule via PEG-derivatization does not preclude the fibrinolytic activity of staphylokinase. While a PEG-molecule appears to reduce the diffusion and therefore fibrinolytic potency of the derivatized staphylokinase within a fibrin clot, dimerization of staphylokinase results in a synergistic fibrinolytic effect on human fibrin clots.

8. Pharmacokinetic properties of dimeric SakSTAR(K109C) and SakSTAR(K102C-PEG) following bolus injection in hamsters

The pharmacokinetic parameters of the disposition of dimeric SakSTAR(K109C) and SakSTAR(K102C-PEG) from blood were evaluated in groups of 4 hamsters following intravenous bolus injection of 100 μg/kg SakSTAR variant. SakSTAR-related antigen was assayed using the ELISA described elsewhere. The ELISA was calibrated against each of the SakSTAR variants to be quantitated. Pharmacokinetic parameters included: initial half-life (in min), $t1/2\alpha = \ln2/\alpha$; terminal half-life (in min), $t1/2\beta = \ln2/\beta$; volume of the central (plasma) compartment (in mL), $VC = dose/(A+B)$; area under the curve (in $\mu g.min.mL^{-1}$), $AUC = A/\alpha + B/\beta$; and plasma clearance (in $mL.min^{-1}$), $Clp = dose/AUC$ (32).

The disposition rate of staphylokinase-related antigen from blood following bolus injection of 100 μg/kg of the selected SakSTAR variants in groups of 4 hamsters could adequately be described by a sum of two exponential terms by graphical curve peeling (results not shown), from which the pharmacokinetic parameters $t1/2\alpha$ and Clp, summarized in Table 19 were derived. The pharmacokinetic parameters of dimeric SakSTAR(K109C) and SakSTAR-(K102C-PEG) were markedly different from those of wild type SakSTAR. Initial plasma half-lives ($t1/2(\alpha)$) were 3.6 and 3.0 min and plasma clearances (Clp) were 0.52 and 0.32 mL/min, for dimeric SakSTAR(K109C) and SakSTAR-(K102C-PEG), respectively. These results may be due to

the increase of the Stokes radius of SakSTAR as a result of the dimerization or crosslinking with PEG. According to size-exclusion chromatography on Superdex50 by HPLC, dimeric SakSTAR(K109C) and SakSTAR(K102C-PEG) have apparent molecular weights of 33 kDa and 40 kDa, respectively.

EXAMPLE 14

Construction, purification and characterization of cysteine-substitution mutants of variants of staphylokinase with reduced immunogenicity

1. Introduction

Based on the results of example 13, additional polyethylene glycol derivatives of SakSTAR variants were constructed, purified and characterized. The least immunogenic variants SakSTAR(E65D,K74R,E80A, D82A,K130T,K135R), (SY19), and SakSTAR(K35A,E65Q, K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K1-30T,K135R,K136A,∇137K), (SY141), were used as templates, with the proviso that the COOH-terminus of the latter was reverted to the wild type sequence, S84A was replaced with E80 and K74Q replaced with K74R, yielding Sak-STAR(K35A,E65Q,K74R,E80A,D82A,T90A,E99D,T101S,E108A, K109A,K130T,K135R), (SY161). The introduced cysteine, which functions as acceptor of the polyethylene glycol molecule was located in the amino terminal region (preferably, but not exclusively, the Ser in position number 3 of the mature staphylokinase variant) in order to be released upon activation of staphylokinase (release of the 10 NH_2-terminal amino acids); finally polyethylene glycol molecules of different molecular weights ($\underline{M}_r$ 5,000 to 20,000) were used, substituted with either OPSS or maleimide.

The mutants described under this example are listed in Table 20. These variants were expressed in E.coli, purified and characterized in terms of specific activity, fibrinolytic properties in human plasma *in vitro*, pharmacokinetic properties following bolus

injection in hamsters, thrombolytic properties following bolus injection in a hamster pulmonary embolism model, and absorption of antibodies from pooled immunized patient plasma (Pool 40).

2. Reagents and Methods

The source of all reagents used in the present study has previously been reported (22), or is specified below. The template vector for mutagenesis, pMEX602sakB (i.e. pMEX.SakSTAR), has been described elsewhere (23). Restriction and modification enzymes were purchased from New England Biolabs (Leusden, The Netherlands), Boehringer Mannheim (Mannheim, Germany) or Pharmacia (Uppsala, Sweden). The enzymatic reactions were performed according to the supplier recommendation. The mutagenic oligonucleotides and primers were obtained from Eurogentec (Seraing, Belgium). Plasmid DNA was isolated using a purification kit from Qiagen (Hilden, Germany), as recommended. Transformation-competent E. coli cells were prepared by the well-known calcium phosphate procedure. Nucleotide sequence determination was performed on double strand plasmid DNA with the dideoxy chain termination method, using the T7 sequencing kit (Pharmacia, Uppsala, Sweden). Polymerase chain reactions (PCR) were performed using Taq polymerase from Boehringer Mannheim (Mannheim, Germany). The recombinant DNA methods required to construct the variants described in this example are well established (22, 27).

3. Construction of expression plasmids

The variants SakSTAR(S3C,E65D,K74R,E80A,D82A, K130T,K135R), (SY19(S3C)), SakSTAR(S2C,S3C,E65D,K74R, E80A,D82A,K130T,K135R), (SY19(2SC,3SC)), SakSTAR(S3C, K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A, K130T,K135R,K136A,∇137K), (SY141(S3C)), SakSTAR(S2C, S3C,K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,-K109A,K130T,K135R,K136A,∇137K), (SY141(S2C,S3C)), Sak-STAR(S3C,K35A,E65Q,K74Q,E80A,D82A,T90A,E99D,T101S,E108A,

K109A,K130T, K135R), (SY160(S3C)) and SakSTAR(S3C,K35A, E65Q,K74R,E80A,D82A,T90A,E99D, T101S,E108A,K109A, K130T,K135R), (SY161(S3C)), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using pMEX.SakSTAR encoding SakSTAR as template, two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the staphylokinase gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC). The forward and backward primers shared an overlap of around 24 bp. The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of pMEX.SakSTAR. For each construction, the sequence of the variant was confirmed by sequencing the entire SakSTAR coding region.

4. <u>Expression and purification of SakSTAR variants</u>

The SakSTAR variants were expressed and purified, as described below, from transformed <u>E. coli</u> grown in terrific broth (TB) medium (28). A 2 to 4 mL aliquot of an overnight saturated culture in LB medium was used to inoculate a 1 to 2 L culture in terrific broth supplemented with 100 μg/mL ampicillin. The culture was incubated with vigorous aeration and at 30°C. After about 16 hours incubation, IPTG (200 μmol/L) was added to the culture to induce expression from the tac promoter. After 3 hours induction, the cells were pelleted by centrifugation at 4,000 rpm for 20 min, resuspended in 1/10 volume of 0.01 mol/L phosphate buffer pH 6-6.5 and disrupted by sonication at 0°C. The suspension was centrifuged for 20 min at 20,000 rpm and the supernatant was stored at 4°C or at -20°C until purification. The

material was purified essentially as described above (Example 2): cleared cell lysates containing the SakSTAR variants were subjected to chromatography on a 1.6 x 5 cm column of SP-Sephadex, followed by chromatography on a 1.6 x 8 cm column of phenyl-Sepharose. The SakSTAR containing fractions, localized by SDS-gel electrophoresis, were pooled for further analysis.

5. Biochemical analysis

Protein concentrations were determined according to Bradford (29). SDS-PAGE was performed with the Phast System™ (Pharmacia, Uppsala, Sweden) using 10-15% gradient gels and Coomassie Brillant blue staining, and the specific activities of SakSTAR solutions were determined with a chromogenic substrate assay carried out in microtiter plates (as described in example 2).

6. Chemical crosslinking of cysteine mutants of SakSTAR with polyethylene glycol

The thiol group of the cysteine mutants was targeted for coupling with an activated polyethylene glycol, either OPSS-PEG or MAL-PEG (Shearwater Polymers Europe, Enschede, The Netherlands). OPSS-PEG is a 5 kDa PEG molecule carrying a single activated thiol group at one end that reacts specifically at slightly alkaline pH with free thiols. MAL-PEG is a 5 kDa, 10 kDa or 20 kDa molecule carrying a maleimide group that reacts specifically with thiol groups under mild conditions in the presence of other functional groups. Modification of the variants was achieved by incubating the molecule (100 μM) with a three-fold excess of OPSS-PEG or MAL-PEG in a 5 mM phosphate, pH 7.9 solution at room temperature. After reaction (about 15 min), the excess of OPSS-PEG or MAL-PEG was removed by purifying the derivatized SakSTAR variant on a 1.6 x 5 cm column of SP-Sephadex as described above (see Example 2). The "pegylated" SakSTAR variant containing fractions, localized by optical densi-

ty at 280 nm, were pooled for further analysis. SDS-PAGE analysis and Coomassie blue staining confirmed that PEG crosslinking was quantitative. As shown in Table 20, the specific activities of the PEG-derivatives were only marginally affected when compared to that of wild-type staphylokinase.

7. Fibrinolytic properties of SakSTAR variants in human plasma in vitro

The fibrinolytic and fibrinogenolytic properties of SakSTAR variants were determined as previously described. Dose- and time-dependent lysis of ^{125}I-fibrin labeled human plasma clots submerged in human plasma was obtained with all molecules tested. Equi-effective concentrations of test compound (causing 50% clot lysis in 2 hrs; C_{50}), determined graphically from plots of clot lysis at 2 hrs versus the concentration of plasminogen activator (not shown), were comparable to or only slightly lower than that of SakSTAR (Table 20). The C_{50} for clot lysis by variants derivatized with P20 (PEG with $\underline{M}_r$ 20 kDa) was about twice as high as the non-derivatized variants. Thus increasing the size of the molecule via PEG-derivatization does not markedly affect the fibrinolytic activity of staphylokinase. The PEG-molecules appear to reduce the diffusion and therefore fibrinolytic potency of the derivatized staphylokinase within a fibrin clot, but this appears to be less pronounced with variants substituted in their NH_2-terminal region - which is released during processing of staphylokinase - than with variants substituted in the core of the molecule (cfr. Tables 19 and 20).

8. Pharmacokinetic properties of SakSTAR variants chemically modified with polyethylene glycol following bolus injection in hamsters

The pharmacokinetic parameters of the disposition of the pegylated variants from blood were evaluated in groups of 4 hamsters following intravenous

bolus injection of 100 $\mu g/kg$ SakSTAR variant. SakSTAR-related antigen was assayed using the ELISA described elsewhere. The ELISA was calibrated against each of the SakSTAR variants to be quantitated. Pharmacokinetic parameters included: initial half-life (in min), $t1/2\alpha = \ln 2/\alpha$; terminal half-life (in min), $t1/2\beta = \ln 2/\beta$; volume of the central (plasma) compartment (in mL), $VC = dose/(A+B)$; area under the curve (in $\mu g.min.mL^{-1}$), $AUC = A/\alpha + B/\beta$; and plasma clearance (in $mL.min^{-1}$), $Clp = dose/AUC$ (32).

The disposition rate of staphylokinase-related antigen from blood following bolus injection of 100 $\mu g/kg$ of the selected SakSTAR variants in groups of 4 hamsters could adequately be described by a sum of two exponential terms by graphical curve peeling (results not shown), from which the plasma clearances Clp, summarized in Table 20 were derived. The clearances of pegylated variants were markedly different from those of wild type SakSTAR and were inversely proportional to the molecular weight of the PEG molecules, with an average reduction of 5-fold with PEG 5 kDa, 10-fold with PEG 10 kDa and 30-fold with PEG 20 kDa. These results may be due to the increase of the Stokes radius of SakSTAR as a result of crosslinking with PEG.

EXAMPLE 15

<u>Comparative thrombolytic efficacy and clearance of Sak-STAR(S3C-P20,E65D,K74R, E80A,D82A,K130T,K135R), (SY19(S3C-P20)), in two patients with acute myocardial infarction</u>

<u>Large scale purification and conditioning of the SakSTAR variant for use in vivo</u>

Material was purified to homogeneity out of culture volumes of 18 liters. The endotoxin content was below 1 IU/mg. Gel filtration on HPLC revealed a single main symmetrical peak in the chromatographic range of the column, representing >98% of the eluted material (total area under the curve) (not shown). SDS gel

electrophoresis of a 30 μg sample revealed single main component. The preparation sterilized by filtration proved to be sterile on 3 day testing as described in methods. Intravenous bolus injection of the SakSTAR variant in 5 mice (3 mg/kg body weight), did not provoke any acute reaction, nor reduced weight gain within 8 days, in comparison with mice given an equal amount of saline (not shown).

Two patients with acute myocardial infarction were given a bolus injection of 5 mg SY19(S3C-P20). These patients had a complete recanalization of the occluded infarct-related artery as determined by coronary angiography at 90 min after the bolus injection. The material was cleared from the plasma with an initial half-life of 3 to 4 hours, as compared to 4 to 6 minutes for wild-type SakSTAR. These data confirm that pegylated variants of SakSTAR may be useful for thrombolytic therapy by single bolus injection at a reduced dose.

CONCLUSION

In summary, the present invention shows that staphylokinase variants with markedly reduced antibody induction but intact thrombolytic potency can be generated. This observation constitutes the first case in which a heterologous protein, with the use of protein engineering techniques, is rendered significantly less immunogenic in man without reducing its biological activity. In addition, the present invention shows that selective chemical modification of staphylokinase or its variants with polyethylene glycol of varying molecular weights is feasible, resulting in a reduction of the plasma clearance proportional to the molecular weight. In the preferred embodiment an amino acid in the NH_2-terminal region of staphylokinase, the portion that is removed by processing, is substituted with Cys and the introduced thiol group is chemically modified with OPSS-PEG or MAL-PEG. This results in homogeneous products which, upon single intravenous bolus injection in experimental

animals and in patients have a maintained thrombolytic potency at markedly reduced doses.

Table 1: Alanine-to-wild-type" reversal variants of "charged-cluster-to-alanine" mutants of SakSTAR: Association constants ($K_A \times 10^7$ mol/L^{-1}) for the binding to insolubilized murine monoclonal antibodies (Mabs), and absorption (percent) of antibodies of immunized patient plasma

Variant	Exp. (mg/L)	Spec. Act. (kU/mg)	murine MAbs Epitope I 17G11	26A2	30A2	2B12	3G10	Epitope II 18F12	14H5	28H4	32B2	7F10	Epitope III 7H11	25E1	40C8	24C4	1A10	SakSTAR patient plasma Pool	Subpool B	Subpool C
SakSTAR		130	22	13	2.9	7.8	11	38	7.4	19	7.7	2.4	4.0	14	5.4	2.9	0.6	95	95	95
SakSTAR(K35A,E38A)		97	15	22	4.2	11	7.9	110	10	15	12	2.2	<0.1	<0.1	<0.1	1.0	1.0	93	91	94
SakSTAR(K74A,E75A,R77A)		110	11	<.01	<0.1	<0.1	<0.1	150	17	28	14	3.3	2.4	11	4.0	2.1	0.9	55	43	95
SakSTAR(K35A,E38A,K74A,E75A,R77A)		50	11	<0.1	<0.1	<0.1	<0.1	110	36	26	15	2.0	<0.1	<0.1	<0.1	1.5	1.2	52	41	92
SakSTAR(E38A,K74A,E75A,R77A)		43	11	<0.1	<0.2	<0.1	<0.1	140	39	26	15	2.1	<0.1	3.2	3.7	1.6	1.1	50	44	95
SakSTAR(K35A, K74A,E75A,R77A)		56	9.2	<0.1	0.15	<0.1	<0.1	52	14	29	8.8	2.3	<0.1	1.8	<0.1	1.8	0.8	46	43	95
SakSTAR(K35A,E38A,E75A,R77A)		44	11	0.3	0.1	0.2	<0.1	75	9.8	12	7.3	1.6	<0.1	<0.1	<0.1	0.53	0.64	92	87	94
SakSTAR(K35A,E38A,K74A,R77A)		41	8.8	2.9	<0.1	2.0	0.33	110	29	31	10	2.0	<0.1	<0.1	<0.1	0.63	0.74	56	50	93
SakSTAR(K35A,E38A,K74A,E75A)		19	13	<0.1	0.1	<0.1	<0.1	180	41	37	15	1.6	<0.1	<0.1	<0.1	1.2	0.45	48	41	92
SakSTAR(E38A,E75A,R77A)		88	11	0.6	0.15	0.4	0.3	79	12	15	10	2.0	<0.1	2.6	4.7	1.1	0.81	95	88	95
SakSTAR(E38A,E75A)		66	16	0.3	<0.1	<0.1	0.9	56	11	13	8.9	2.0	<0.1	20	4.8	1.3	1.6	91	90	95
SakSTAR(K35A,E75A,R77A)		68	9.2	<0.1	<0.1	<0.1	<0.1	60	7.0	13	11	3.3	<0.1	1.5	<0.1	0.8	1.1	88	89	95
SakSTAR(K35A,E75A)		150	17	0.12	<0.1	0.16	0.14	40	7.2	13	9.2	4.2	<0.1	1.8	<0.1	1.4	1.5	94	93	95
SakSTAR(K74A)		100	12	7.6	0.17	4.4	2.1	55	15	33	14	3.6	2.9	14	4.9	3.4	1.2	59	45	95
SakSTAR(E75A)		140	13	1.2	<0.1	<0.1	<0.1	46	8.5	14	12	3.4	4.5	18	5.0	1.2	2.1	95	93	95
SakSTAR(K74A,E75A,R77A,E80A,D82A)		50	14	<0.1	<0.1	<0.1	<0.1	180	19	33	19	3.7	<0.1	<0.1	<0.1	<0.1	1.2	49	29	89
SakSTAR(E80A,D82A)		130	7.3	12	2.1	6.5	5.9	79	6.1	8.4	7.8	1.9	<0.1	<0.1	<0.1	<0.1	0.44	89	83	92
SakSTAR(E80A)		160	13	13	3.3	7.9	10	35	7.4	17	8.6	2.1	<0.1	16	3.6	<0.1	1.7	94	93	95
SakSTAR(D82A)		160	17	12	4.8	7.3	11	31	7.8	17	12	2.7	<0.1	0.18	<0.1	<0.1	2.3	95	93	95
SakSTAR(E75A,D82A)		170	20	15	3.1	6.6	7.2	69	8.1	15	14	4.9	0.17	0.7	0.5	0.1	1.4	95	95	95

Apparent association constants ≥ 10-fold lower than those of wild-type SakSTAR are represented in bold type; Spec. Act. ≥ 100.000 HU/mg represented in bold type; ≤60% absorption represented in bold type.

Table 2: **Baseline characteristics and treatment outcome of the patients with peripheral arterial occlusion treated with SakSTAR, SakSTAR(K74A) or SakSTAR(K74A,E75A,R77A)**

Compound Patient Id.	Gender	Age (yrs)	Clinical ischemia	Locus of occlusion	Age of occlusion (days)	Length of occlusion (cm)	Recanalization by thrombolysis	Total dose of thrombolytic agent (mg)	Total duration of infusion (hrs)	Additional therapy
SakSTAR										
MEE	F	67	Rest pain	Left SFA	30	8	complete	7.0	5.0	PTA
FOR	M	68	Claudication	Left IA (stent)	14	18	complete	6.5	4.5	PTA + stent
DAN	M	73	Claudication	Right SFA	30	6	complete	7.5	5.5	PTA
BER	F	63	Rest pain	Left FT graft	18	55	complete	18	28	PTA
DAM	F	43	Acute	Left brachial and radial artery	2	7	complete	19	17	PTA + stent
TOR	M	68	Claudication	Right SFA (popliteal aneurysm)	50	12	complete	6.0	4.0	PTA + femoropopliteal bypass graft
CLA	M	74	Acute	Left PA	1.5	20	complete	9.0	7.0	-
MAN	M	65	Acute	Left EIA (stent)	4	20	complete	6.5	4.5	(amputation left digit V)
MAT	M	64	Subacute	Right FP graft	3	45	complete	8.0	6.0	(-)
Mean ± SEM		65 ± 3.0			17 ± 5.6	21 ± 5.8		9.7 ± 1.7	9.1 ± 2.7	
SakSTAR(K74A)										
LIE	M	70	Subacute	Right FF graft	10	48	complete	11	9.0	PTA
ENG	M	50	Claudication	Right SFA	28	10	complete	12	10	PTA
COX	F	48	Claudication	Right PA graft	25	7	partial	15	15	PTA
MAN	F	68	Claudication	Right SFA	≥120	9	complete	9.0	7.0	PTA
VHE	M	47	Acute	Right IF graft	10	54	complete	18	16	Surgical graft revision
MUL	F	51	Acute	Right IF and FP graft	1	63	complete	16	20	PTA
BUR	F	67	Rest pain	Right TF trunc	9.0	38	partial	18	21	-
NIJ	F	60	Rest pain	Left AF graft	23	78	complete	15	21	-
POE*	M	49	Subacute	Right TF trunc	2	30	partial	6.0	4.0	rt-PA, surgical graft lengthening
VBE	M	39	Subacute	Right BA (embolism)	20	28	complete	18	23	Stent right SC artery, first rib resection
SME	F	50	Subacute	TF trunc	18	32	complete	21	19	None
WOL	M	67	Subacute	Right PA	4	25	complete	16	22	-
Mean ± SEM		56 ± 3.0			23 ± 9.2	35 ± 6.4		15 ± 1.2	16 ± 1.9	
SakSTAR(K74A,E75A,R77A)										
JAC	F	65	Acute	Right BA and UA	0.3	5	complete	14	12	-
MAE	M	74	Rest pain	Left SFA	10	50	complete	9.0	7.0	PTA
CRA	F	52	Claudication	Right IA and FA artery	14	28	complete	25	23	PTA + stent
VDB	M	68	Claudication	Left SFA	90	12	complete	9.0	7.0	PTA
DUN	M	71	Subacute	Left SFA	14	6	complete	9.0	7.0	PTA
DEL	M	59	Acute	Right FT graft	3	42	complete	9.0	7.0	PTA
Mean ± SEM		65±3.3			22±14	24±7.8		13 ± 2.6	11 ± 2.6	

AF: aortofemoral; BA: brachial artery; CIA: common iliac artery; FF: femorofibular; FP: femoropopliteal; FT: femorotibial; IA: iliac artery; IF: iliofemoral; PA: popliteal artery; PTA, percutaneous transluminal angioplasty; SFA: superficial femoral artery; TF: tibiofibular; UA: ulnar artery. *Previous treatment with SakSTAR in 1994

Table 3: Alanine-substitution variants of SakSTAR: Association constants ($K_A \times 10^7 mol/L^{-1}$) for binding to insolubilized murine monoclonal antibodies (Mab) and absorption (percent) of antibodies of immunized patient plasma

Variant	Exp. (mg/L)	Spec. Act. (kU/mg)	murine MAbs															SakSTAR patient plasma		
			Epitope cluster I					Epitope cluster II					Epitope cluster III							
			17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool	Subpool B	Subpool C
SakSTAR		120	9.3	13	2.9	7.8	11	38	7.4	19	7.7	2.4	4.0	14	5.4	2.9	0.6	95	95	95
			16.4	17	7.4	19	35	18	25	>18	>14	1.1	0.6	11	16	6.3	2.0			
SakSTAR(S34G,G36R,H43R)		120	10	14	3.3	7.5	11	<0.1	<0.1	<0.1	20	2.7	<0.1	<0.1	<0.1	0.15	1.7	87	76	75
SakSTAR(F4A)	LE																			
SakSTAR(D5A,K6A)		150	11.3	21	12	9.2	9.7	12	23	17	10	0.4	1.3	3.9	0.8	4.5	3.8	95	95	95
SakSTAR(K8A,K10A)		24	11.8	16	5.1	29	15	22	16	26	18	1.0	0.93	11	1.1	18	0.75	95	95	95
SakSTAR(Y9A)	24	78	22	49	6.6	2.3	16	44	8.4	20	14	2.6	2.4	16	9.6	1.1	0.5	96	95	95
SakSTAR(K11A,D13A,D14A)	LE																			
SakSTAR(D13A)	6	46	2.4	6.1	2.0	3.7	3.4	11	1.9	4.5	4.4	8.7	1.5	2.4	1.1	3.6	<0.1	95	94	95
SakSTAR(D14A)	14	30	5.1	13	4.0	6.6	3.1	38	7.7	12	15	2.2	2.7	6.0	3.2	5.6	<0.1	95	94	95
SakSTAR(S16A)	41	160	8.1	16	4.5	8.4	9.0	15	6.1	13	21	3.6	4.0	8.0	4.3	2.5	0.5	95	95	95
SakSTAR(Y17A,F18A)	27	30	13	22	3.3	10	9.5	21	4.6	6.7	12	2.5	1.2	18	6.5	3.4	<0.1	95	95	95
SakSTAR(E19A,P20A)	36	9	11	19	3.3	9.2	12	15	6.1	11	16	1.0	1.1	15	5.1	3.3	<0.1	93	93	95
SakSTAR(T21A)	54	170	4.8	15	2.4	8.7	9.6	32	11	24	18	1.3	1.8	9.6	2.9	5.6	0.6	95	95	95
SakSTAR(P23A)	41	67	14	31	4.4	14	22	41	5.3	37	13	10	4.0	11	7.6	3.1	1.9	91	95	95
SakSTAR(Y24A)	10	40	17	33	4.3	13	11	33	4.3	7.0	12	4.0	0.4	14	6.8	4.8	<0.1	95	95	95
SakSTAR(L25A)	LE																			
SakSTAR(M26A)	LE																			
SakSTAR(V27A)	62	50	3.3	15	1.6	7.8	7.4	12	2.9	3.7	33	2.0	1.3	5.9	2.8	4.2	1.2	95	95	95
SakSTAR(N28A)	90	<5	5.8	29	2.1	7.0	5.5	27	10	20	2.5	2.1	2.1	5.6	2.1	2.1	0.7	95	95	95
SakSTAR(N28A,V29A)	32	45	18	30	2.5	20	18	20	14	20	24	2.7	3.3	20	1.1	5.0	2.0	93	95	95
SakSTAR(T30A)	52	140	7.4	13	2.1	7.0	6.1	7.6	3.4	5.1	13	3.3	5.6	12	3.4	5.4	0.8	94	95	95
SakSTAR(V32A)	78	45	10	9.6	2.4	6.2	7.8	56	17	12	14	1.4	<0.1	<0.1	<0.1	<0.1	2.2	90	93	95
SakSTAR(D33A,K35A)		130	12.1	19	14	14	19	15	24	32	10	5.3	1.4	5.1	3.8	5.1	3.0	95	95	95
SakSTAR(S34A)	29	110	17	24	4.6	9.5	11	28	11	22	15	2.9	3.1	8.8	3.8	2.0	0.2	95	95	93

Table 3 - cont'd: Alanine-substitution variants of SakSTAR: Association constants (K_A x $10^7 mol/L^{-1}$) for binding to insolubilized murine monoclonal antibodies (Mab) and absorption (percent) of antibodies of immunized patient plasma

Variant	Exp. (mg/L)	Spec. Act. (kU/mg)	murine MAbs															SakSTAR patient plasma		
			Epitope cluster I					Epitope cluster II					Epitope cluster III							
			17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool	Subpool B	Subpool C
SakSTAR(K35A)		230	6.4	14	3.3	8.0	7.4	31	11	12	11	2.6	<0.1	1.3	0.2	1.7	0.8	91	88	95
SakSTAR(K35A,E38A)		97	15	22	4.2	11	7.9	110	10	15	12	2.2	<0.1	<0.1	<0.1	1.0	1.0	93	91	94
SakSTAR(G36A)	14	72	3.5	9.8	1.5	5.7	6.5	52	4.2	17	9.2	1.4	<0.1	<0.1	0.9	5.0	1.0	86	83	78
SakSTAR(N37A)	40	110	5.6	31	3.0	10	11	20	4.1	14	10	2.9	1.4	5.3	3.5	3.6	0.8	95	95	95
SakSTAR(L39A,L40A)	8	<5	14	15	3.1	5.1	8.0	27	16	6.3	12	2.7	1.2	5.4	3.2	2.1	0.9	93	93	95
SakSTAR(S41A,P42A)	24	48	10	25	4.1	13	12	11	3.0	1.9	27	2.7	3.2	15	4.8	3.6	1.1	95	95	95
SakSTAR(H43A)	37	69	15	28	9.7	18	7.6	<0.1	<0.1	<0.1	9.1	1.5	2.0	23	7.8	7.2	1.6	95	95	95
SakSTAR(H43A,Y44A)	13	<5	10	22	3.7	17	15	<0.1	<0.1	<0.1	1.4	3.0	2.3	11	5.2	2.1	0.1	95	95	95
SakSTAR(V45A)	19	<5	16	5.6	1.4	4.8	6.3	2.2	0.2	1.7	32	2.6	2.1	8.3	1.1	2.8	1.6	91	92	95
SakSTAR(E46A,K50A)	LE																			
SakSTAR(F47A)	6	<5	<0.1	4.0	1.0	3.9	3.4	5.7	2.7	2.8	8.5	1.7	0.9	8.8	3.0	3.0	0.9	90	82	97
SakSTAR(I49A)	2	43	2.7	27	7.8	23	22	35	4.4	11	6.2	1.1	2.0	5.7	2.0	1.7	0.6	95	95	95
SakSTAR(K50A)	15	42	<0.1	13	2.9	7.8	8.7	46	8.3	12	2.2	0.5	2.8	6.4	4.0	2.3	0.6	95	94	95
SakSTAR(T53A,T54A)	14	68	0.9	19	2.7	7.6	7.8	41	6.7	12	15	1.5	1.9	5.1	2.3	1.0	0.6	93	94	95
SakSTAR(L55A)	LE																			
SakSTAR(T56A)	17	150	5.5	15	3.2	12	13	56	5.3	12	11	2.0	3.5	6.1	2.7	4.3	1.2	94	92	95
SakSTAR(K57A,E58A,K59A)		94	14	8.7	6.0	7.3	27	16	14	6.7	5.6	0.52	0.36	1.7	0.42	1.0	1.1|			
SakSTAR(I60A)	11	96	12	20	2.9	11	13	27	6.4	25	2.7	1.5	0.7	5.8	2.9	1.7	1.0	95	95	95
SakSTAR(E61A,E65A)		80	19.5	>10	8.8	21	29	>11	>16	6.6	>7.2	4.6	0.5	4.6	2.0	5.9	1.5]			
SakSTAR(Y62A,Y63A)	24	<5	<0.1	4.3	0.3	2.1	1.9	11	2.2	3.1	8.4	1.7	0.6	9.2	3.6	3.8	0.7	89	83	95
SakSTAR(Y63A)	4	<5	<0.1	18	3.7	9.6	13	17	5.3	3.3	15	1.1	2.2	5.3	4.3	1.0	3.7	89	82	95
SakSTAR(V64A)	14	48	14	16	2.9	6.3	7.8	15	15	21	21	2.6	1.6	7.6	5.6	2.8	0.7	94	92	95
SakSTAR(E65A)	25	97	53	20	4.4	12	7.0	10	5.6	9.7	1.9	1.8	2.3	4.7	3.0	5.8	0.97	95	95	95
SakSTAR(E65A,D69A)		<5																		
SakSTAR(W66A)	26	<5	<0.1	<0.1	<0.1	<0.1	<0.1	15	4.4	5.7	23	3.3	2.0	10	1.4	1.8	0.8	85	78	92
SakSTAR(L68A)	16	93	14	22	3.5	8.5	9.3	29	8.7	16	15	4.0	2.1	5.3	3.6	4.1	<0.1	92	92	95
SakSTAR(T71A)	LE																			

Table 3 - cont'd: **Alanine-substitution variants of SakSTAR: Association constants ($K_A \times 10^7 mol/L^{-1}$) for binding to insolubilized murine monoclonal antibodies (Mab) and absorption (percent) of antibodies of immunized patient plasma**

Variant	Exp. (mg/L)	Spec. Act. (kU/mg)	murine MAbs															SakSTAR patient plasma		
			Epitope cluster I					Epitope cluster II					Epitope cluster III							
			17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool	Subpool B	Subpool C
SakSTAR(Y73A)	20	<5	9.5	<0.1	<0.1	<0.1	4.8	33	7.3	11	8.9	2.0	0.5	9.0	2.5	1.4	0.8	63	44	93
SakSTAR(Y73A,K74A)	24	<5	18	<0.1	<0.1	<0.1	<0.1	19	6.7	23	9.9	3.2	2.7	13	4.0	1.6	1.1	47	28	87
SakSTAR(K74A)	80	69	4.4	2.7	0.2	2.2	1.1	17	5.2	14	7.6	2.2	2.0	6.8	3.3	1.8	0.9	64	58	95
SakSTAR(K74A,E75A,R77A)		68	9.2	<0.1	<0.1	<0.1	<0.1	60	7.0	13	11	3.3	<0.1	1.5	<0.1	0.8	1.1	88	89	95
SakSTAR(K74A,R77A)	34	41	3.5	1.8	0.2	1.5	0.4	20	2.4	10	2.1	1.8	1.7	2.3	2.2	1.2	0.7	74	60	95
SakSTAR(E75A)		140	13	1.2	<0.1	<0.1	<0.1	46	8.5	14	12	3.4	4.5	18	5.0	1.2	2.1	95	93	95
SakSTAR(F76A)	9	90	20	9.6	1.0	2.7	3.9	13	6.2	20	15	1.7	0.3	5.9	2.1	1.2	1.0	94	92	95
SakSTAR(V78A,V79A)	23	68	12	23	4.0	10	17	21	18	34	28	2.3	1.6	4.7	<0.1	0.5	1.7	93	93	95
SakSTAR(E80A)		160	13	13	3.3	7.9	10	35	7.4	17	8.6	2.1	<0.1	16	3.6	<0.1	1.7	94	93	95
SakSTAR(E80A,D82A)		130	7.3	12	2.1	6.5	5.9	79	6.1	8.4	7.8	1.9	<0.1	<0.1	<0.1	<0.1	0.4	89	83	92
SakSTAR(L81A)	23	28	12	33	1.6	40	11	52	11	17	17	3.9	1.4	5.2	7.1	4.6	1.5	88	95	95
SakSTAR(D82A)		160	17	12	4.8	7.3	11	31	7.8	17	12	2.7	<0.1	0.2	<0.1	<0.1	2.3	95	93	95
SakSTAR(D82A,S84A)	72	130	8.3	14	2.6	8.1	8.5	23	3.8	12	11	1.7	<0.1	<0.1	1.4	<0.1	1.0	91	91	95
SakSTAR(S84A)	12/26	89	8.0	16	3.8	8.6	10	90	8.3	11	36	1.8	2.2	1.6	3.0	3.5	0.5	95	95	95
SakSTAR(K86A,E88A)		73	17.2	1.4	3.7	6.0	4.6	5.7	4.9	7.7	15	4.4	<0.1	5.4	0.80	1.9	0.13			
SakSTAR(I87A)	18	98	6.7	23	2.8	8.6	9.1	10	3.6	11	7.4	2.7	1.1	7.8	3.4	4.5	1.0	95	95	95
SakSTAR(V89A)	20	87	4.6	11	2.6	6.6	2.2	28	7.2	7.3	3.0	1.3	1.2	5.1	2.9	3.1	0.83	95	95	95
SakSTAR(T90A)	78	120	6.0	12	0.9	3.7	3.1	20	4.8	7.2	<0.1	<0.1	2.1	6.6	2.6	2.1	0.5	95	95	95
SakSTAR(Y91A)	5	53	6.0	16	3.0	7.0	13	28	8.2	16	0.6	2.1	1.4	3.7	1.6	1.6	0.2	95	95	95
SakSTAR(Y92A)	16	120	16	23	4.1	13	12	29	7.3	18	<0.1	1.7	4.4	10	3.9	5.9	1.1	95	95	95
SakSTAR(E93A,K94A)		97	18.2	19	13	30	24	18	11	>10	9.0	0.88	1.4	11	2.4	7.0	2.11			
SakSTAR(K94A,N95A,K97A)	32	8	NT															95	94	95
SakSTAR(N95A)	25	260	10	18	4.0	10	11	50	13	14	4.9	2.3	3.7	7.3	4.7	2.9	0.8	95	94	95
SakSTAR(K96A,K97A,K98A)		47	12.8	41	23	37	90	>16	9.1	19	16	0.41	0.58	17	1.2	13	0.30			
SakSTAR(E99A)	24	42	7.4	15	4.0	8.4	8.9	22	2.7	4.7	<0.1	<0.1	2.1	6.2	7.3	14	0.8	92	91	92
SakSTAR(E99A,E100A)	I.E																			
SakSTAR(T101A)	23	85	4.6	11	2.1	6.6	7.3	30	2.8	16	0.7	1.0	1.3	5.4	2.4	2.9	0.8	95	95	95

Table 3 - cont'd: Alanine-substitution variants of SakSTAR: Association constants ($K_A \times 10^7 mol/L^{-1}$) for binding to insolubilized murine monoclonal antibodies (Mab) and absorption (percent) of antibodies of immunized patient plasma

Variant	Exp. (mg/L)	Spec. Act. (kU/mg)	murine MAbs: Epitope cluster I					Epitope cluster II					Epitope cluster III					SakSTAR patient plasma		
			17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool	Subpool B	Subpool C
SakSTAR(K102A)	12	89	4.9	12	3.7	6.5	6.3	30	3.1	15	8.2	6.1	0.8	4.2	1.9	10	0.6	95	93	95
SakSTAR(S103A)	67	210	9.0	16	5.0	9.4	9.1	19	5.9	13	13	3.6	3.9	8.3	4.7	2.8	0.9	94	95	95
SakSTAR(F104A)	14	55	5.8	19	4.8	14	27	7.3	5.0	14	4.8	<0.1	0.4	7.6	3.4	11	1.3	95	93	95
SakSTAR(I106A)	2	93	2.3	13	3.0	7.4	6.7	5.5	5.2	17	11	1.4	1.8	3.1	1.8	1.2	0.5	95	95	95
SakSTAR(T107A)	32	130	5.2	15	3.4	9.8	10	32	8.7	4.7	14	1.9	3.1	6.3	3.2	5.0	0.8	94	94	95
SakSTAR(E108A,K109A)		170	11.6	5.1	7.2	19	5.1	28	15	21	21	1.2	0.43	6.9	1.4	10	1.9			
SakSTAR(F111A)	5	49	3.7	16	3.8	13	22	21	8.4	12	3.1	0.8	2.8	2.9	1.5	1.5	0.9	95	95	95
SakSTAR(V112A,V113A)	64	130	4.2	16	3.9	10	12	34	5.8	13	8.0	0.3	1.5	4.3	2.3	3.0	0.8	95	95	95
SakSTAR(D115A,S117A)	80	54	3.3	14	4.1	15	15	17	3.4	19	0.7	<0.1	1.5	4.8	2.6	1.3	0.9	95	95	95
SakSTAR(D115A,E118A,H119A)		32	12.5	32	3.4	21	8.7	13	9.9	23	9.3	1.2	1.0	24	2.1	9.0	1.8			
SakSTAR(L116A,S117A)	25	<5	4.4	35	3.6	33	42	160	29	220	<0.1	<0.1	0.5	4.1	4.9	3.5	1.6	94	95	95
SakSTAR(H119A,K121A)		130	18.0	24	11	26	29	25	14	29	12	0.52	1.2	11	2.9	20	1.2			
SakSTAR(I120A)	26	75	23	26	5.1	17	16	30	9.8	25	9.0	6.9	3.0	15	5.1	5.2	1.0	93	95	95
SakSTAR(N122A)	5	19	NT															95	93	95
SakSTAR(F125A)	3	<10	2.8	18	4.7	11	18	17	3.2	6.0	1.9	<0.1	0.3	5.3	2.1	0.9	1.6	93	90	95
SakSTAR(N126V)	11	51	7.6	13	2.0	12	13	30	58	290	9.8	2.5	1.8	8.0	4.2	6.5	0.7	95	95	95
SakSTAR(L127A)	11	54	8.9	6.7	1.8	5.0	6.6	25	4.9	14	8.4	1.5	0.9	1.9	0.9	2.5	1.8	93	94	95
SakSTAR(I128A)	10	20	16	25	4.8	15	14	38	2.6	4.3	8.2	2.9	2.5	2.0	4.2	6.7	0.9	95	93	95
SakSTAR(T129A)	44	190	5.3	15	2.3	14	24	21	13	15	4.2	2.3	0.7	10	3.3	1.3	1.0	95	95	95
SakSTAR(K130A)	130	280	5.1	12	3.2	6.4	3.5	22	6.7	11	15	2.7	<0.1	<0.1	4.1	0.9	0.6	92	74	71
SakSTAR(V131A)	130	70	6.5	17	2.9	11	13	19	14	19	29	3.4	1.9	13	5.3	8.6	0.9	95	95	95
SakSTAR(V132A)	100	130	4.2	15	2.6	9.2	11	33	12	30	19	2.1	2.1	3.6	<0.1	2.6	0.4	95	95	95
SakSTAR(I133A)	3	99	9.4	15	1.9	7.8	7.8	24	6.0	9.1	8.6	1.4	0.56	6.4	1.6	1.6	0.9	95	95	95
SakSTAR(E134A,K135A,K136A)		74	122	21	6.7	25	25	>18	>25	>15	>12	1.7	0.2	11	0.94	6.0	2.61			
SakSTAR(K135A)	54	410	5.2	12	11	7.9	11	20	11	11	3.8	2.0	1.6	6.9	3.7	1.9	0.9	95	95	95
SakSTAR(K136A)		180	7.6	18	5.6	12	13	54	5.3	12	16	3.2	3.5	8.6	3.9	1.8	0.5	91	83	95

LE: expression level below 3 mg/L

Table 4: Mutagenesis of S34, G36 and H43: Association constants ($K_A \times 10^7 mol/L^{-1}$) for binding to insolubilized murine monoclonal antibodies (Mab) and absorption (percent) of antibodies of immunized patient plasma

Variant	Exp.	Spec. Act.	murine MAbs															SakSTAR patient plasma		
			Epitope cluster I					Epitope cluster II					Epitope cluster III							
	(mg/L)	(kU/mg)	17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool	Subpool B	Subpool C
SakSTAR		120	9.3	13	2.9	7.8	11	38	7.4	19	7.7	2.4	4.0	14	5.4	2.9	0.6	95	95	95
SakSTAR(S34G,G36R,H43R)		120	10	14	3.3	7.5	11	<0.1	<0.1	<0.1	20	2.7	<0.1	<0.1	<0.1	0.15	1.7	87	76	75
SakSTAR(S34A)	29	110	17	24	4.6	9.5	11	28	11	22	15	2.9	3.1	8.8	3.8	2.0	0.2	95	95	93
SakSTAR(G36A)	14	72	3.5	9.8	1.5	5.7	6.5	52	4.2	17	9.2	1.4	<0.1	<0.1	0.9	5.0	1.0	86	83	78
SakSTAR(G36E)	12	66	3.1	8.7	1.4	4.8	4.7	12	2.8	6.1	7.6	1.0	<0.1	<0.1	<0.1	3.4	1.1	89	83	72
SakSTAR(G36K)	34	88	9.9	23	3.1	8.3	9.8	21	3.9	13	15	3.0	<0.1	<0.1	<0.1	2.6	1.2	88	80	69
SakSTAR(G36L)	15	92	3.6	11	1.8	6.1	6.1	16	1.4	6.4	12	1.3	<0.1	<0.1	<0.1	0.6	1.1	93	85	72
SakSTAR(G36N)	10	91	8.7	10	1.6	6.1	6.2	33	3.3	7.8	7.9	1.8	<0.1	<0.1	1.0	0.3	0.5	86	80	75
SakSTAR(G36Q)	21	92	10	12	1.8	6.7	6.5	23	3.8	7.5	7.3	1.5	<0.1	<0.1	<0.1	0.1	0.4	87	84	73
SakSTAR(G36R)	45	100	11	2.4	3.3	10	10	27	4.6	14	20	3.4	<0.1	<0.1	<0.1	3.1	1.2	89	81	70
SakSTAR(H43A)	37	69	15	28	9.7	18	7.6	<0.1	<0.1	<0.1	9.1	1.5	2.0	23	7.8	7.2	1.6	95	95	95
SakSTAR(H43R)	43	120	15	11	2.7	7.6	11	<0.1	<0.1	<0.1	13	6.4	0.7	18	6.7	5.7	1.4	95	95	95
SakSTAR(S34G,G36R)	45	90	3.1	12	2.3	4.8	4.2	13	8.3	24	9.1	1.9	<0.1	<0.1	<0.1	<0.1	0.6	92	83	69
SakSTAR(S34G,G36R,H43R,K74A)	1	12	12	4.7	3.8	7.4	9.4	<0.1	0.1	0.6	25	2.3	<0.1	<0.1	0.5	0.8	1.7	67	56	83
SakSTAR(S34G,G36R,K74A)	15	26	4.0	2.1	<0.1	1.8	0.6	10	2.2	15	13	1.8	<0.1	<0.1	<0.1	0.3	2.2	59	28	68
SakSTAR(K35G,G36R,H43D)	32	6	1.8	1.5	1.6	2.0	8.9	<0.1	<0.1	<0.1	7.1	1.3	<0.1	<0.1	<0.1	<0.1	0.9	82	75	72
SakSTAR(G36R,K74A)	40	35	19	7.0	0.2	4.3	2.0	53	27	28	19	4.4	<0.1	<0.1	<0.1	1.2	1.0	48	33	58
SakSTAR(G36R,K74R)	68	150	4.7	17	3.8	11	8.0	16	6.0	6.4	3.0	1.6	<0.1	<0.1	<0.1	0.2	0.8	81	54	73
SakSTAR(G36R,K74A,N95A)	11	25	6.1	2.9	2.9	2.4	<0.1	31	5.7	12	5.3	4.1	<0.1	<0.1	<0.1	<0.1	0.9	53	32	63
SakSTAR(G36R,K74A,K135R)	20	33	5.8	3.4	<0.1	1.7	0.7	26	16	14	17	1.2	<0.1	<0.1	<0.1	0.4	0.5	64	32	68
SakSTAR(G36R,K74R,K135R)	48	75	6.1	17	5.8	10	3.3	31	14	13	5.7	2.3	<0.1	<0.1	<0.1	0.3	0.8	77	49	68

Table 5: Mutagenesis of K35, Y73, K74, E80/D82, N95, K130, V132 and K135: Association constants (K_A x $10^7 mol/L^{-1}$) for binding to insolubilized murine monoclonal antibodies (Mab) and absorption (percent) of antibodies of immunized patient plasma

Variant	Exp. (mg/L)	Spec. Act. (kU/mg)	murine MAbs: Epitope cluster I					Epitope cluster II					Epitope cluster III					SakSTAR patient plasma		
			17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool	Subpool B	Subpool C
SakSTAR		120	9.3	13	2.9	7.8	11	38	7.4	19	7.7	2.4	4.0	14	5.4	2.9	0.6	95	95	95
SakSTAR(S34G,G36R,H43R)		120	10	14	3.3	7.5	11	<0.1	<0.1	<0.1	20	2.7	<0.1	<0.1	<0.1	0.15	1.7	87	76	75
SakSTAR(K35A)		230	6.4	14	3.3	8.0	7.4	31	11	12	11	2.6	<0.1	1.3	0.2	1.7	0.8	91	88	95
SakSTAR(K35E)	75	160	4.8	10	0.6	2.7	2.7	21	5.7	9.1	4.8	1.0	<0.1	<0.1	<0.1	1.5	0.4	95	95	92
SakSTAR(K35Q)	9	69	3.2	9.5	1.3	5.2	5.4	22	2.7	9.3	8.5	1.2	0.5	1.7	<0.1	1.9	1.0	95	95	95
SakSTAR(Y73A)	20	<5	9.5	<0.1	<0.1	<0.1	4.8	33	7.3	11	8.9	2.0	0.5	9.0	2.5	1.4	0.8	63	44	93
SakSTAR(Y73F)	6	31	7.9	12	1.3	2.1	8.0	24	15	19	15	2.6	1.6	6.2	3.5	7.2	1.4	93	95	95
SakSTAR(Y73H)	30	<5	7.3	1.5	<0.1	<0.1	1.4	20	7.5	17	42	4.3	3.5	6.8	5.9	9.7	1.3	76	65	95
SakSTAR(Y73L)	33	<5	11	<0.1	<0.1	<0.1	<0.1	84	19	26	53	3.4	4.2	7.1	4.2	3.4	1.0	81	60	94
SakSTAR(Y73S)	31	<5	7.0	3.5	0.59	0.6	3.0	21	8.3	14	21	3.2	2.2	6.7	1.3	1.3	0.7	86	69	95
SakSTAR(Y73W)	6	27	4.8	9.0	4.6	4.6	11	8.4	4.5	11	8.0	2.7	2.9	5.0	3.0	3.8	1.3	73	53	93
SakSTAR(K74A)	80	69	4.4	2.7	0.2	2.2	1.1	17	5.2	14	7.6	2.2	2.0	6.8	3.3	1.8	0.9	64	58	95
SakSTAR(K74E)	12	<5	2.2	0.6	<0.1	0.7	0.1	14	1.8	7.5	9.3	1.2	2.0	3.0	1.2	0.6	1.0	83	43	90
SakSTAR(K74N)	9	39	2.9	4.7	1.1	3.3	1.7	10	1.6	4.8	13	1.5	1.9	4.0	1.8	1.4	0.9	63	46	95
SakSTAR(K74Q)	64	110	5.3	5.8	<0.1	2.5	1.1	24	5.9	12	5.4	1.1	2.0	6.2	2.3	2.0	0.4	70	62	94
SakSTAR(K74R)	44	150	2.1	7.5	2.0	4.1	4.2	24	6.9	8.0	8.3	1.3	2.2	7.8	3.2	2.1	0.5	75	70	95
SakSTAR(E80A,D82A)		130	7.3	12	2.1	6.5	5.9	79	6.1	8.4	7.8	1.9	<0.1	<0.1	<0.1	<0.1	0.4	89	83	92
SakSTAR(E80A)		160	13	13	3.3	7.9	10	35	7.4	17	8.6	2.1	<0.1	16	3.6	<0.1	1.7	94	93	95
SakSTAR(D82A)		160	17	12	4.8	7.3	11	31	7.8	17	12	2.7	<0.1	0.2	<0.1	<0.1	2.3	95	93	95
SakSTAR(N95A)	25	260	10	18	4.0	10	11	50	13	14	4.9	2.3	3.7	7.3	4.7	2.9	0.8	95	94	95
SakSTAR(N95E)	12	79	2.8	8.5	1.3	5.2	5.4	17	2.7	10	5.2	1.1	0.5	4.0	1.7	1.8	0.6	95	92	95
SakSTAR(N95G)	20	160	4.1	11	1.5	6.8	7.6	36	3.3	15	3.6	1.5	0.7	5.8	2.7	2.7	0.9	95	90	95
SakSTAR(N95K)	54	180	9.5	14	3.2	9.0	11	51	5.0	18	4.8	2.5	1.6	8.3	2.9	4.8	1.1	95	95	95
SakSTAR(N95R)	LE																			

Table 5 - cont'd: Mutagenesis of K35, Y73, K74, E80/D82, N95, K130, V132 and K135: Association constants (K_A x $10^7 mol/L^{-1}$) for binding to insolubilized murine monoclonal antibodies (Mab) and absorption (percent) of antibodies of immunized patient plasma

Variant	Exp.	Spec. Act.	murine MAbs															SakSTAR patient plasma		
			Epitope cluster I					Epitope cluster II					Epitope cluster III							
	(mg/L)	(kU/mg)	17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool	Subpool B	Subpool C
SakSTAR(K130A)		280	5.1	12	3.2	6.4	3.5	22	6.7	11	15	2.7	<0.1	<0.1	4.1	0.9	0.6	92	74	71
SakSTAR(K130T)		290	7.8	14	3.1	8.0	5.6	43	5.4	9.7	17	2.7	<0.1	<0.1	3.4	0.5	0.5	92	84	82
SakSTAR(V132A)	102	130	4.2	15	2.6	9.2	11	33	12	30	19	2.1	2.1	3.6	<0.1	2.6	0.4	95	95	95
SakSTAR(V132L)	126	120	4.8	14	2.3	8.0	9.7	67	10	48	20	2.5	2.0	11	<0.1	4.8	0.4	95	95	95
SakSTAR(V132T)	78	140	4.5	13	2.4	7.8	9.0	39	10	25	16	2.0	1.1	2.0	<0.1	<0.1	0.4	95	95	95
SakSTAR(V132N)	16	150	4.5	11	1.7	7.0	7.2	21	17	20	15	2.0	1.5	1.3	<0.1	4.2	0.7	95	95	95
SakSTAR(V132R)	76	230	5.4	12	0.8	3.3	3.2	23	5.5	7.8	4.8	1.1	2.1	3.4	<0.1	0.4	0.4	95	95	95
SakSTAR(K135A)	54	410	5.2	12	11	7.9	11	20	11	11	3.8	2.0	1.6	6.9	3.7	1.9	0.9	95	95	95
SakSTAR(K135F)	68	64	3.9	6.3	1.0	6.1	4.1	13	4.5	8.4	16	2.1	1.6	5.8	1.9	1.5	0.9	95	95	95
SakSTAR(K135R)	54	230	4.0	14	1.4	9.3	5.0	31	8.1	13	23	1.9	2.4	2.4	2.6	2.1	0.5	95	95	95
SakSTAR(K35A,K74A)	20	130	NT															84	50	95
SakSTAR(Y73A,K74A)	24	<5	18	<0.1	<0.1	<0.1	<0.1	19	6.7	23	9.9	3.2	2.7	13	4.0	1.6	1.1	47	28	87
SakSTAR(Y73F,K74A)	21	6	17	6.7	<0.1	2.1	<0.1	42	19	21	20	4.9	1.9	2.8	6.6	2.1	0.8	51	34	90
SakSTAR(I60A,K74A,N95A)		84	5.3	2.7	<0.1	2.5	5.3	17	7.2	9.6	3.4	2.3	2.1	6.2	2.7	2.9	0.8	60	47	95
SakSTAR(N95A,K135R)	120	240	4.9	13	1.9	9.0	9.9	18	12	15	2.5	1.5	1.8	5.9	3.6	3.7	0.8	95	95	95
SakSTAR(K130T,K135R)	15	280	3.7	10	1.6	7.2	8.0	13	7.7	4.5	3.7	1.6	<0.1	<0.1	2.4	0.4	0.6	89	60	73

Table 6: Combination mutants of SakSTAR(K130T,K135R) with K35A, G36R, E65X,K74X and selected other amino acids

Variant	Exp. (mg/mL)	Spec. Act. (kU/mg)	murine MAbs: Epitope cluster I					Epitope cluster II					Epitope cluster III					SakSTAR patient plasma				Code
			17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool 10	Subpool B	Subpool C	Pool 40	
SakSTAR(K130T,K135R)	15	280	3.7	10	1.6	7.2	8.0	13	7.7	4.5	3.7	1.6	<0.1	<0.1	2.4	0.4	0.6	89	60	73	90	SY2
SakSTAR(G36R,K130T,K135R)	26	220	7.2	18	2.8	11	14	17	3.9	8.9	5.1	1.6	<0.1	<0.1	<0.1	<0.1	1.1	79	65	69	-	SY3
SakSTAR(K74R,K130T,K135R)	18	310	7.3	27	5.9	16	18	17	3.7	11	5.1	1.5	<0.1	<0.1	3.2	0.7	0.9	76	49	69	78	SY4
SakSTAR(K74Q,K130T,K135R)	64	190	4.0	7.2	3.0	7.7	0.9	20	5.7	7.2	8.4	1.8	<0.1	<0.1	2.7	0.7	1.0	50	25	67	62	SY41
SakSTAR(G36R,K74R,K130T,K135R)	5	210	7.6	26	5.7	17	19	17	4.7	10	5.0	1.4	<0.1	<0.1	<0.1	<0.1	0.8	73	44	69	75	SY5
SakSTAR(G36R,K74Q,K130T,K135R)	88	120	5.5	7.3	0.8	11	6.4	35	11	7.1	6.1	2.6	<0.1	<0.1	<0.1	<0.1	0.8	51	25	63	54	SY42
SakSTAR(G36R,H43R,K74R,K130T,K135R)	29	160	5.6	8.8	2.2	10	10	<0.1	<0.1	<0.1	6.3	2.0	<0.1	<0.1	<0.1	<0.1	0.9	72	39	69	-	SY9
SakSTAR(S34G,G36R,K74Q,K130T,K135R)	40	76	4.8	5.9	0.5	13	1.5	18	8.7	7.5	5.1	2.1	<0.1	<0.1	<0.1	<0.1	1.1	52	25	65	61	SY43
SakSTAR(E65A,K74Q,K130T,K135R)	46	170	11	9.0	2.3	11	13	19	66	12	5.7	2.3	<0.1	<0.1	3.5	1.2	0.6	45	16	77	55	SY48
SakSTAR(G36R,E65A,K74Q,K130T,K135R)	80	83	4.7	12	1.2	16	21	27	12	10	6.9	2.6	<0.1	<0.1	<0.1	<0.1	0.7	44	18	65	46	SY44
SakSTAR(G36R,E65A,K74A,K130A,K135R)	17	71	5.7	10	1.8	13	12	21	8.4	6.9	4.6	1.8	<0.1	<0.1	<0.1	<0.1	1.0	41	14	64	50	SY59
SakSTAR(E65A,A72S,K74Q,K130T,K135R)	60	96	5.6	6.0	2.0	3.6	4.9	21	8.6	8.8	2.8	2.5	<0.1	<0.1	3.5	1.7	1.1	51	13	66	56	SY51
SakSTAR(E65Q,K74Q,K130T,K135R)	40	150	6.7	18	2.1	15	16	9.0	3.1	4.1	6.3	2.3	<0.1	<0.1	3.8	0.9	0.6	53	29	67	65	SY49
SakSTAR(K74Q,K86A,K130T,K135R)	54	130	2.4	4.9	<0.1	7.4	3.8	19	8.7	7.6	4.7	1.9	<0.1	<0.1	3.5	1.3	1.5	56	32	69	61	SY55
SakSTAR(E65Q,T71S,K74Q,K130T,K135R)	32	210	6.2	13	1.8	10	13	11	3.9	5.0	68	23	<0.1	<0.1	3.1	1.1	0.8	49	21	64	59	SY65
SakSTAR(E65Q,K74Q,E75A,K130T,K135R)	36	46	7.7	<0.1	<0.1	<0.1	<0.1	10	3.9	3.2	7.4	2.6	<0.1	<0.1	4.6	1.3	0.6	43	15	62	55	SY66
SakSTAR(E65Q,K74Q,E75D,K130T,K135R)	35	67	7.0	<0.1	<0.1	<0.1	<0.1	13	5.4	4.9	6.6	2.5	<0.1	<0.1	3.2	1.3	1.0	49	29	63	57	SY67
SakSTAR(K74Q,K130T,K135R,K136A,+137A)	19	78	4.3	24	<0.1	2.7	5.6	20	9.6	7.5	5.6	2.2	<0.1	<0.1	1.7	1.1	1.2	37	12	57	50	SY68
SakSTAR(K74Q,K130A,K135R)	28	240	5.6	5.4	0.5	7.5	5.3	21	8.4	14	6.1	2.4	<0.1	<0.1	4.1	2.6	0.7	57	27	78	65	SY56
SakSTAR(E65Q,K74Q,K130A,K135R)	60	230	6.0	14	2.4	15	17	9.0	3.3	5.8	6.3	2.3	<0.1	<0.1	4.3	2.0	0.6	51	32	73	58	SY69
SakSTAR(K74Q,K130E,K135R)	46	300	4.1	4.4	0.8	6.6	3.8	18	8.5	7.8	5.2	2.1	<0.1	<0.1	2.4	0.7	0.9	55	29	64	59	SY57
SakSTAR(E65Q,K74Q,K130A,K135A)	88	170	5.3	8.9	1.7	7.7	11	16	3.4	5.8	6.2	2.4	<0.1	<0.1	3.5	2.4	0.7	55	27	79	55	SY70
SakSTAR(K74Q,K130E,V132R,K135R)	68	170	4.5	4.9	0.4	6.0	5.2	9.0	4.9	4.3	5.6	2.4	<0.1	<0.1	<0.1	<0.1	0.6	51	20	63	56	SY58
SakSTAR(E65Q,K74Q,T90A,K130A,K135R)	36	170	6.2	13	1.8	12	14	7.3	2.5	3.8	<0.1	<0.1	<0.1	<0.1	4.1	1.9	0.5	51	27	69	57	SY71

Table 6 - cont'd: Combination mutants of SakSTAR(K130T,K135R) with K35A, G36R, E65X,K74X and selected other amino acids

Variant	Exp. mg/L	Spec. Act. (kU/mg)	murine MAbs															SakSTAR patient plasma				Code
			Epitope cluster I					Epitope cluster II					Epitope cluster III									
			17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool 10	Subpool B	Subpool C	Pool 40	
SakSTAR(E65Q,K74Q,N95A,K130A,K135R)	40	220	6.1	14	1.9	17	15	9.0	3.3	5.8	6.3	2.3	<0.1	<0.1	4.1	2.2	0.5	52	29	74	58	SY72
SakSTAR(E65Q,K74Q,E118A,K130A,K135R)	86	180	8.5	18	2.8	15	27	11	4.1	5.7	7.3	2.6	<0.1	<0.1	6.1	2.8	0.5	50	28	72	58	SY73
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R)	33	190	7.8	18	2.4	7.7	21	20	3.9	6.1	6.6	2.3	<0.1	<0.1	5.8	2.5	0.5	48	27	74	58	SY74
SakSTAR(N95A,K130A,K135R)	85	410	6.1	11	3.3	18	15	37	5.9	9.6	6.8	2.5	<0.1	<0.1	4.5	3.0	0.6	93	81	82	94	INT1
SakSTAR(K35A,E65Q,K74Q,K130A,K135R)	29	110	NT															49	26	63	45	SY75
SakSTAR(K35A,H43R,E65Q,K74Q,K130A,K135R)		14	NT															49	23	73	55	SY76
SakSTAR(E65Q,K74Q,S103A,K130A,K135R)	32	60	6.7	15	2.6	14	16	8.0	2.7	3.9	6.3	2.3	<0.1	<0.1	4.6	1.6	0.6	55	27	75	61	SY77
SakSTAR(T21A,K35A,E65Q,K74Q,K130A,K135R)		110	NT															50	26	72	50	SY78
SakSTAR(T56A,E65Q,K74Q,K130T,K135R)		180	NT															52	31	61	55	SY79
SakSTAR(K57A,E58A,E61A,K74Q,K130T,K135R)		120	NT															57	24	61	54	SY80
SakSTAR(E65Q,K74Q,K109A,K130T,K135R)	40	210	7.3	15	2.1	12	12	14	2.5	4.0	5.8	2.3	<0.1	<0.1	3.4	1.8	0.7	50	22	68	51	SY81
SakSTAR(E65Q,K74Q,E108A,K130T,K135R)		120																51	24	61	54	SY82
SakSTAR(E65Q,K74Q,E108A,K109A,K130T,K135R)	62	180	9.3	13	1.4	13	17	17	3.0	4.1	6.8	2.5	<0.1	<0.1	3.7	2.6	0.5	55	21	67	50	SY83
SakSTAR(E65Q,K74Q,K121A,K130T,K135R)	73	150	5.7	13	1.5	11	14	22	3.1	4.6	1.2	<0.1	<0.1	<0.1	3.5	1.8	0.9	61	25	69	57	SY85
SakSTAR(E19A,E65Q,K74Q,K130T,K135R)		3	NT															51	27	62	56	SY86
SakSTAR(E65Q,K74Q,D115A,K130T,K135R)		57	NT															52	25	62		SY87
SakSTAR(G36R,E65A,K74Q,K130E,V132R,K135R)	48	60	7.6	9.9	1.4	11	14	42	19	17	4.3	1.0	<0.1	<0.1	<0.1	<0.1	0.9	44	17	70	44	SY60
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,+137A)		120																45	30	74	60	SY93
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,+137K)		1,400																37	16	70	54	SY94

Association constants ≥ 10-fold lower and antibody absorption ≤60 percent of wild-type SakSTAR are represented in bold type; ≥ 100,000 HU/mg represented in bold type. NT: not tested.

Table 7: Combination mutants of SakSTAR(E80A,D82A,K130T,K135R) with K35A, G36R, E65X, K74X, and selected other amino acids

Variant	Exp. (mg/mL)	Spec. Act. (kU/mg)	murine MAbs: Epitope cluster I					Epitope cluster II					Epitope cluster III					SakSTAR patient plasma				Code
			17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool 10	Subpool B	Subpool C	Pool 40	
SakSTAR(E80A,D82A,K130T,K135R)	20	250	8.4	17	3.1	11	13	18	4.3	11	5.5	1.5	<0.1	<0.1	<0.1	<0.1	1.0	80	64	68		SY6
SakSTAR(K74R,E80A,D82A,K130T,K135R)	4	220	5.3	31	2.8	18	11	89	8.4	21	5.9	1.8	<0.1	<0.1	<0.1	<0.1	0.6	74	34	69	72	SY7
SakSTAR(K74Q,E80A,D82A,K130T,K135R)	27	110	5.1	6.5	1.2	7.9	7.3	28	6.4	19	4.6	1.7	<0.1	<0.1	<0.1	<0.1	0.8	46	17	60	48	SY15
SakSTAR(K35A,K74R,E80A,D82A,K130T,K135R)	70	160	5.9	5.7	6.4	2.7	18	19	16	8.2	9.0	1.8	<0.1	<0.1	<0.1	<0.1	0.8	66	34	66	68	SY17
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	22	140	5.4	50	2.9	49	21	31	11	37	3.4	1.8	<0.1	<0.1	<0.1	<0.1	0.6	43	11	68	57	SY19
SakSTAR(E65S,K74R,E80A,D82A,K130T,K135R)	3	110	3.2	12	4.6	44	15	19	5.0	15	6.6	1.4	<0.1	<0.1	<0.1	<0.1	0.4	35	12	60	-	SY20
SakSTAR(E65T,K74R,E80A,D82A,K130T,K135R)	10	94	7.2	9.3	9.9	5.6	32	22	15	8.2	11	2.0	<0.1	<0.1	<0.1	<0.1	1.0	58	24	69	-	SY21
SakSTAR(S34G,G36R,K74R,K130T,K135R)		250	5.6	54	2.9	38	25	36	6.5	18	5.5	1.9	<0.1	<0.1	<0.1	<0.1	0.9	75	33	68	-	SY10
SakSTAR(E65A,K74R,E80A,D82A,K130T,K135R)	40	140	8.2	10	13	5.7	55	24	22	11	15	2.2	<0.1	<0.1	<0.1	<0.1	1.0	51	17	66	-	SY18
SakSTAR(E65N,K74R,E80A,D82A,K130T,K135R)	88	120	8.5	12	11	7.0	36	43	13	12	7.9	2.4	<0.1	<0.1	<0.1	<0.1	0.9	60	29	67	-	SY23
SakSTAR(E65Q,K74R,E80A,D82A,K130T,K135R)	55	140	9.0	16	16	9.9	59	18	4.5	4.3	10	2.5	<0.1	<0.1	<0.1	<0.1	0.8	54	22	63	60	SY22
SakSTAR(K57A,E58A,E61A,E80A,D82A,K130T,K135R)	24	110	2.4	17	2.9	13	12	16	7.8	16	4.5	1.6	<0.1	<0.1	<0.1	<0.1	0.7	75	64	62	74	SY13
SakSTAR(E65A,A72S,K74R,E80A,D82A,K130T,K135R)	92	62	8.2	23	3.8	8.4	19	30	11	13	3.6	3.1	<0.1	<0.1	<0.1	<0.1	1.2	51	13	66	52	SY53
SakSTAR(E65D,K74Q,E80A,D82A,K130T,K135R)	84	110	7.0	3.9	2.6	4.1	7.2	29	28	14	6.8	2.2	<0.1	<0.1	<0.1	<0.1	0.9	43	13	64	42	SY30
SakSTAR(E65Q,K74Q,E80A,D82A,K130T,K135R)	54	120	5.1	16	3.2	14	3.7	16	20	2.4	5.7	1.7	<0.1	<0.1	<0.1	<0.1	1.0	43	21	64	42	SY47
SakSTAR(K35A,E65D,K74Q,E80A,D82A,K130T,K135R)	56	140	5.1	6.8	2.6	9.5	1.7	28	17	14	8.4	2.1	<0.1	<0.1	<0.1	<0.1	1.0	35	8	58	40	SY46

Table 7 - cont'd: Combination mutants of SakSTAR(E80A,D82A,K130T,K135R) with K35A, G36R, E65X, K74X, and selected other amino acids

Variant	Exp. (mg/mL)	Spec. Act. (kU/mg)	murine MAbs: Epitope cluster I					murine MAbs: Epitope cluster II					murine MAbs: Epitope cluster III					SakSTAR patient plasma				Code
			17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool 10	Subpool B	Subpool C	Pool 40	
SakSTAR(K74R,E80A,D82A,S103A,K130T,K135R)	32	160	4.9	22	5.8	14	4.3	26	6.9	7.4	5.0	1.7	<0.1	<0.1	<0.1	<0.1	0.9	67	32	69	70	SY24
SakSTAR(K35A,E65D,K74R,E80A,D82A,E108A,K109A,K130T,K135R)	9.0	89	5.8	30	2.6	26	10	13	16	13	3.2	1.8	<0.1	<0.1	<0.1	<0.1	0.5	55	10	63	47	SY12
SakSTAR(K35A,E65D,K74R,E80A,D82A,E108A,K130T,K135R)	20	91	6.4	20	5.0	15	3.9	22	17	7.4	2.4	1.9	<0.1	<0.1	<0.1	<0.1	0.9	44	8	70	53	SY32
SakSTAR(E65D,K74R,E80A,D82A,E108A,K130T,K135R)	4	90	8.1	6.7	6.9	4.3	29	19	31	11	14	2.1	<0.1	<0.1	<0.1	<0.1	1.0	52	11	69	-	SY33
SakSTAR(K35A,E65D,K74R,E80A,D82A,K109A,K130T,K135R)	42	84	5.5	18	5.3	14	1.6	18	12	7.7	10	1.7	<0.1	<0.1	<0.1	<0.1	1.0	43	6	61	50	SY36
SakSTAR(E65D,K74R,E80A,D82A,K109A,K130T,K135R)	60	130	9.7	6.6	6.8	4.2	28	11	32	12	17	2.3	<0.1	<0.1	<0.1	<0.1	0.9	56	10	64	53	SY37
SakSTAR(K35A,E65D,K74R,E80A,D82A,K130T,K135R,K136A)	28	81	4.5	12	3.3	11	1.7	22	13	7.6	4.9	1.6	<0.1	<0.1	<0.1	<0.1	0.8	40	14	52	40	SY34
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R,K136A)	60	100	6.8	5.8	4.4	4.5	15	33	32	14	7.9	2.0	<0.1	<0.1	<0.1	<0.1	0.8	46	28	67	45	SY35
SakSTAR(E65Q,K74Q,D82A,S84A,K130T,K135R)		170	NT															45	21	60	45	SY50N
SakSTAR(K35A,E65D,K74R,E80A,D82A,K86A,K130T,K135T)	68	86	4.4	20	5.5	15	1.5	15	12	6.4	6.7	1.9	<0.1	<0.1	<0.1	<0.1	1.0	36	7	60	55	SY40
SakSTAR(K35A,K74Q,E80A,D82A,K130T,K135R)	72	120	6.1	3.4	2.5	3.0	5.9	38	14	9.8	6.8	1.9	<0.1	<0.1	<0.1	<0.1	0.8	49	16	64	48	SY28
SakSTAR(K35A,E65D,K74R,E80A,D82A,K130T,K135R)	54	190	8.1	7.5	6.9	5.5	25	37	34	14	7.7	2.3	<0.1	<0.1	<0.1	<0.1	1.0	56	28	68	55	SY29
SakSTAR(K35A,E65D,K74R,E80A,D82A,V132R,K135R)	13	55	6.7	23	5.3	17	2.3	47	19	19	5.1	2.0	<0.1	<0.1	<0.1	<0.1	1.1	53	20	88	62	SY61
SakSTAR(K35A,E65D,K74R,E80A,D82A,T129A,K135R)	13	61	7.0	13	5.1	31	12	27	12	11	6.7	2.5	<0.1	<0.1	<0.1	<0.1	1.9	56	18	79	60	SY62
SakSTAR(K35A,E65D,K74R,E80A,D82A,T129A,K135A)	23	21	6.9	27	5.8	32	20	29	6.6	9.7	5.4	2.1	<0.1	<0.1	<0.1	<0.1	0.9	56	17	91	60	SY64

Association constants ≥ 10-fold lower and antibody absorption ≤60 percent of wild-type SakSTAR are represented in bold type; ≥ 100,000 HU/mg represented in bold type. NT: not tested.

Table 8: SakSTAR variants with intact specific activity (≥ 100 kHU/mg) and ≤50 percent absorption of human antibodies elicited by treatment with wild-type SakSTAR

Variant	Spec. Act. (kU/mg)	SakSTAR patient plasma				Code
		Pool 10	Subpool B	Subpool C	Pool 40	
SakSTAR(K74Q,K130T,K135R)	190	50	25	67	62	SY41
SakSTAR(E65A,K74Q,K130T,K135R)	170	45	16	77	55	SY48
SakSTAR(E65Q,T71S,K74Q,K130T,K135R)	210	49	21	64	59	SY65
SakSTAR(E65Q,K74Q,E118A,K130A,K135R)	180	50	28	72	58	SY73
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R)	190	48	27	74	58	SY74
SakSTAR(K35A,E65Q,K74Q,K130A,K135R)	110	49	26	63	45	SY75
SakSTAR(E65Q,K74Q,K109A,K130T,K135R)	210	50	22	68	51	SY81
SakSTAR(K74Q,E80A,D82A,K130T,K135R)	110	46	17	60	48	SY15
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	140	43	11	68	57	SY19
SakSTAR(E65S,K74R,E80A,D82A,K130T,K135R)	110	35	12	60	-	SY20
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R,K136A)	100	46	28	67	45	SY35
SakSTAR(K35A,K74Q,E80A,D82A,K130T,K135R)	120	49	16	64	48	SY28
SakSTAR(E65D,K74Q,E80A,D82A,K130T,K135R)	110	43	13	64	42	SY30
SakSTAR(E65Q,K74Q,E80A,D82A,K130T,K135R)	120	43	21	64	42	SY47
SakSTAR(E65Q,K74Q,D82A,S84A,K130T,K135R)	170	45	21	60	45	SY50N
SakSTAR(K35A,E65D,K74Q,E80A,D82A,K130T,K135R)	140	35	8	58	40	SY46
SakSTAR(T21A,K35A,E65Q,K74Q,K130A,K135R)	110	50	26	72	50	SY78
SakSTAR(E65Q,K74Q,K109A,K121A,K130A,K135R)	140	50	31	73	52	SY88
SakSTAR(E65Q,K74Q,D82A,S84A,K109A,K130A,K135R)	180	43	20	62	44	SY89

<u>Table 8 - cont'd</u>: **SakSTAR variants with intact specific activity (≥ 100 kHU/mg) and ≤50 percent absorption of human antibodies elicited by treatment with wild-type SakSTAR**

Variant	Spec. Act. (kU/mg)	SakSTAR patient plasma				Code
		Pool 10	Subpool B	Subpool C	Pool 40	
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,∇137A)	**120**	**45**	**30**	74	**60**	SY93
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,∇137K)	**1,400**	**37**	**16**	70	**54**	SY94
SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130A,K135R)	**110**	**46**	**26**	63	**41**	SY95

Antibody absorption ≤60 percent of wild-type SakSTAR are represented in bold type; ≥ 100,000 HU/mg represented in bold type.

Table 9: Fibrinolytic properties of selected SakSTAR variants in human plasma in vitro

Compound	Fibrinolytic potency (C50 in µg/mL)	Residual fibrinogen at C50 (% of baseline)	Fibrinogenolytic potency (C50 in µg/mL)	Code
SakSTAR	0.18 ± 0.01	93 ± 3.5	24 ± 3.6	
SakSTAR(K74Q,E80A,D82A,K130T,K135R)	0.15 ± 0.01	97 ± 3.0	14 ± 3.2	SY15
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	0.24 ± 0.04	94 ± 10	29 ± 3.1	SY19
SakSTAR(K35A,E65D,K74Q,E80A,D82A,K130T,K135R)	0.11 ± 0.01	92 ± 3.0	20 ± 2.0	SY46
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,∇137K)	0.13	91		SY93

The data represent mean ± SD of 3 experiments.

C_{50}: amount of wild type or variant SakSTAR required for 50% clot lysis or 50% fibrinogen breakdown in 2 hrs.

Table 10: **Pharmacokinetic parameters of the disposition of staphylokinase-related antigen from plasma following bolus injection of SakSTAR variants (100 µg/kg) in hamsters.**

Variant	C_0 (µg/mL)	A (µg/mL)	B (µg/mL)	t1/2 (α) (min)	t1/2 (β) (min)	V_C (mL)	AUC (µg.min.mL^{-1})	Cl_p (mL.min^{-1})
SakSTAR	0.8 ± 0.1	0.6 ± 0.1	0.2 ± 0.0	2.8	7.0	13 ± 1.0	4.6 ± 0.4	2.2 ± 0.2
SakSTAR(K74Q,E80A,D82A,K130T,K135R)	0.5 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	2.0	10	20 ± 2.2	2.5 ± 0.3	4.1 ± 0.5
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	0.6 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	2.0	10	16 ± 1.1	2.8 ± 0.2	3.7 ± 0.3
SakSTAR(K35A,E65DK74Q,E80A,D82A,K130T,K135R)	1.1 ± 0.1	1.0 ± 0.1	0.1 ± 0.0	2.0	24	9.6 ± 0.7	6.4 ± 0.5	1.6 ± 0.1

Data are mean ± SEM of 4 experiments.

Table 11: Baseline characteristics and treatment outcome of the patients with peripheral arterial occlusion treated with SakSTAR, SakSTAR(K74Q,E80A,D82A,K130T,K135R) or SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)

Compound Patient Id.	Gender	Age (yrs)	Clinical ischemia	Locus of occlusion	Age of occlusion (days)	Length of occlusion (cm)	Recanalization by thrombolysis	Total dose of thrombolytic agent (mg)	Total duration of infusion (hrs)	Additional therapy
SakSTAR										
PUT	M	66	Subacute	Femoro-femoral graft	6	5	Complete	2	23	Stenting left IF artery
VERM	M	73	Acute	Right PA	2	6	Partial	13	23	Right upper leg amputation
GEIV	V	63	Restpain	Left SFA	10	5	Complete	8	7	PTA
POL	M	46	Subacute	Right SFA	30	50	Partial	22	29	Lumbal sympathectomie
BUE	F	53	Claudication	Right AF graft	1	15	Complete	10	13	Desobstruction
VIJ	F	75	Subacute	Left FT graft	2	34	Complete	7	10	PTA
REN	M	48	Restpain	Right IF graft	4	20	Complete	6.5	5	Left AF graft
COR	V	78	Acute	Left AFS	14	9	Complete	4	3	PTA
MAN	M	67	Restpain	Left tibial artery	1	6	Partial	6	5	-
STRA	M	66	Claudication	Right FP graft	14	16	Complete	19	26	-
VANH	M	38	Acute	Left radial artery	4	1	Complete	6	5	-
VANW	F	57	Acute	Right FP graft	1	25	Complete	20	24	New right FP graft
BRA	M	57	Acute	Left FT graft	1	30	Complete	25	43	-
DON	M	60	Claudication	Left FT graft	1	20	Complete	13	19	PTA + stenting
CAM	M	77	Restpain	Right SFA graft	8	30	Complete	27	44	FF graft
Mean ± SEM		62 ± 3.1			6.6 ± 2.1	18 ± 3.5		13 ± 2.1	19 ± 3.5	
SakSTAR(K74Q,E80A,D82A,K130T,K135R)										
IMB	M	66	Claudication	Left SFA	30	5	Complete	24	24	PTA
AZY	M	44	Subacute	Right C.I.A.	7	8	Complete	18	23	Stenting
VIN	M	51	Acute	Right E.I.A.	5	70	Complete	24	30	-
STRO	M	53	Claudication	Left FP junction	14	5	Partial	3.5	2	Aspiration thrombectomy, PTA
VERG	M	62	Restpain	Left SFA	20	6	Complete	19	25	FP bypass
GIE	M	76	Acute	Right FP bypass	2	15	Complete	8.5	17	-
Mean ± SEM		59 ± 4.7			13 ± 4.3	18 ± 10		16 ± 3.4	20 ± 4.0	
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)										
URB	M	57	Subacute	Right E.I.A.	4	8	Complete	8	6	Pseudo aneurysm, right AF graft revision
COM	M	59	Restpain	Right AF graft	7	65	Complete	16	22	-
HAC	M	70	Restpain	Left anterior tibial artery	7	15	Complete	12	14	
DEW	F	76	Restpain	SFA	21	6	Complete	6	4	-
VAI	F	65	Subacute	Left PA	25	10	Partial	8	6	Aspiration thrombectomy
FIL	M	76	Claudication	Right SFA	28	8	Complete	24	31	PTA
Mean ± SEM		67 ± 3.4			15 ± 4.3	19 ± 9.4		12 ± 2.8	14 ± 4.4	

AF: aortofemoral; CABG: coronary artery bypass graft; CAD, coronary artery disease; CIA: common iliac artery; COPD: chronic obstructive pulmonary disease; DM: diabetes mellitus; EIA: external iliac artery; FF: femorofibular; FP: femoropopliteal; FT: femorotibial; IA: iliac artery; IF: iliofemoral; occl: occlusion; PA: popliteal artery; PTA: percutaneous transluminal angioplasty; SFA: superficial femoral artery; TA: tibial artery; TF: tibiofibular; SC: subclavian.

Table 12: Absorption with SakSTAR variants of antibodies elicited with SakSTAR variants in patients with peripheral arterial occlusion

Treatment	Absorbant	Insolubilized compound		
		SakSTAR	SakSTAR(K74Q,E80A,D82A,K130T,K135R)	SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)
SakSTAR (Pool 40)				
	SakSTAR	95		
	SakSTAR(K74Q,E80A,D82A,K130T,K135R)	48		
	SalSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	57		
SakSTAR(K74Q,E80A,D82A,K130T,K135R) (Imb., Vin., Ver., Gie.)				
	SakSTAR	94	95	95
	SakSTAR(K74Q,E80A,D82A,K130T,K135R)	91	93	89
	SalSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	92	94	94
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) (Urb.)				
	SakSTAR	90	88	85
	SakSTAR(K74Q,E80A,D82A,K130T,K135R)	94	95	94
	SalSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	94	95	94

Data represent median values of the percent absorption with 250 nM absorbant, measured by residual binding to insolubilized compound.

Table 13: Additive substitution mutagenesis of SakSTAR(E65Q,K74Q,K130T, K135R) with selected other amino acids

Variant	Spec. Act. (kU/mg)	Antibody absorption (percent)	Code
SakSTAR(E65Q,K74Q,K130T,K135R)	**150**	65	SY49
SakSTAR(E65Q,K74Q,D82A,S84A,K130T,K135R)	**170**	**45**	SY50
SakSTAR(E65Q,K74Q,T90A,E99D,T101S,K130A,K135R)	**410**	**51**	SY98
SakSTAR(E65Q,K74Q,E108A,K109A,K130T,K135R)	**180**	**50**	SY83
SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R)	**110**	**41**	SY95
SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R,K136A,∇137K)	**1,500**	**30**	SY118
SakSTAR(E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K)	**2,900**	**28**	SY128
SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K)	**3,700**	**24**	SY141
SakSTAR(K35A,E65Q,K74R,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K)	**5,700**	**31**	SY145

Spec. Act. ≥ 100 kU/mg is represented in bold type. Absorption of antibodies (in percent) from pooled immunized patient plasma; values ≤60% are represented in bold type.

Table 14: Fibrinolytic properties of SakSTAR variants in human plasma in vitro

Compound	Fibrinolytic potency (C50 in µg/ml)	Residual fibrinogen at C50 (% of baseline)	Fibrinogenolytic potency (C50 in µg/ml)	Code
SakSTAR	0.18 ± 0.01	93 ± 3.5	24 ± 3.6	
SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R,K136A,∇137K)	0.15 ± 0.02	90 ± 5.0	14 ± 1.0	SY118
SakSTAR(K35A,E65Q,K74Q,D82A,S84A;T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K)	0.17 ± 0.01	87 ± 3.0	7 ± 0.6	SY141
SakSTAR(K35A,E65Q,K74R,D82A,S84A;T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K)	0.19 ± 0.01	82 ± 3.0	7 ± 0.9	SY145

The data represent mean ± SD of 3 experiments.
C50: amount of wild type or variant SakSTAR required for 50% clot lysis or 50% fibrinogen breakdown in the absence of fibrin in 2 hrs.

Table 15: **Characteristics of the patients with peripheral arterial occlusion treated with SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R,K136A,∇137K), SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇ 137K) or SakSTAR(K35A,E65Q,K74R,D82A,S84A,T90A,E99D,T101S, E108A,K109A,K130T,K135R,K136A,∇137K)**

Compound Patient Id.	Gender	Age (yrs)	Clinical ischemia	Risk factors Relevant history	Current Smoking	Locus of occlusion	Age of occlusion (days)	Length of occlusion (cm)
SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R,K136A,∇137K) (SY118)								
VCL	M	69	Acute	Hypertension, ischemic heart disease, ABF graft	-	Left AF graft	10	14
REN	M	76	Subacute	Hypercholesterolemia	-	Right PA	18	14
HOL	M	69	Acute	Hypertension, hypercholesterolemia, right bypass	+	Right FT bypass	16	3.0
PAR	M	79	Pain, swelling	-	-	Left popliteal to communal femoral vein	50	8.0
FRA	M	60	Subacute	Ischemic heart disease, left FP graft	+	Left FP graft	30	14
MAC	V	73	Acute	Hypertension, ABF graft		Left branch ABF graft	2.0	10
Mean ± SEM		71 ± 2.7					21 ± 6.9	11 ± 1.8
SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K) (SY141)								
VERH	F	52	Claudication	Hypertension, hypercholesterolemia, right IF endoprothesis	+	Right IA	14	12
DUB	M	54	Claudication	Hypertension, stenting left, right IA	+	Right EIA	30	18
VAP	M	46	Claudication	Hypertension, hypercholesterolemia, stenting left + right IA	-	Aortabifurcation	21	25
WYN	M	43	Claudication	CAD; hypercholesterolemia; stenting left FP graft	+	Left FP graft	5.0	30
HOR	M	57	Acute	Hypertension; left FP graft	+	Left CIA, left FP graft	7.0	>60
AND	M	75	Acute	Diabetes; hypertension; cardiac valve replacements	-	Left SF artery	3.0	10
Mean ± SEM		55 ± 4.6					13 ± 4.3	19 ± 3.5
SakSTAR(K35A,E65Q,K74R,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K) (SY145)								
LIN	F	48	Restpain	Hypertension, ischemic heart disease	+	Right SF artery	7.0	16
DEL	M	68	Claudication	Hypertension	+	Left PA	21	6.0
LAM	M	71	Acute	FP graft	-	Right FP graft	7.0	26
BAS	M	64	Acute	ABF graft, ischemic heart disease, hypertension	-	Right SFA	3.0	6.0
TOU	M	68	Acute	Ischemic heart disease, hypertension	+	Right PA	1,0	6.0
Mean ± SEM		64 ± 4.1					7.8 ± 3.5	12 ± 4.0

ABF: Aortobifemoral; AF: aortofemoral; CABG: coronary artery bypass grafting; CAD, coronary artery disease; CIA: common iliac artery; COPD: chronic obstructive pulmonary disease; DM: diabetes mellitus; EIA: external iliac artery; FF: femorofibular; FP: femoropopliteal; FT: femorotibial; IA: iliac artery; IF: iliofemoral; PA: popliteal artery; SFA: superficial femoral artery; TF: tibiofibular; AMI: acute myocardial infarction.

Table 16: Treatment and outcome in patients with peripheral arterial occlusion, treated with SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R,K136A,∇137K), SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K) or SakSTAR(K35A,E65Q,K74R,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T, K135R,K136A,∇137K)

Compound Patient Id.	Recanalization by thrombolysis	Total dose of thrombolytic agent (mg)	Total duration of infusion (hrs)	Additional therapy	Complications and remarks
SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R,K136A,∇137K) (SY118)					
VCL	Complete	17	22	None	Puncture site hematoma
REN	Complete	24	22	None	Puncture site hematoma
HOL	Complete	14	9.0	None	None
PAR	Partial (normal patency with residual thrombi after first control)	10	6.0	-	Small subdural hematoma
FRA	Complete	24	22	New FP graft	None
MAC	Complete	8.0	6.0	None	Brain stem hemorrhage; death
Mean ± SEM		16 ± 2.8	15 ± 3.4		
SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K) (SY141)					
VERH	Complete	15	18	Femorofemoral cross over	-
DUB	Complete	6.0	4.0	-	-
VAP	Complete	14	22	RIA-stenting, bilateral IA stenting	Puncture site hematoma
WYN	Complete	23	29	FP graft revision	None
HOR	Complete	10	8.0	None	None
AND	Complete	13	17	None	Retroperitoneal hematoma, died due to septicemia
Mean ± SEM		14 ± 2.3	16 ± 3.7		
SakSTAR(K35A,E65Q,K74R,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K) (SY145)					
LIN	Complete	14	24	None	Retroperitoneal hematoma, Hemorragies
DEL	Complete	7.0	5.0	None	None
LAM	Complete	23	30	None	Puncture site hematoma
BAS	Complete			None	
TOU					
Mean ± SEM		15 ± 4.6	20 ± 7.5		

PTA. percutaneous transluminal angioplasty; IF: iliofemoral; FT: femorotibial; FP: femoropopliteal.

Table 17: Neutralizing antibody activity before and after administration of SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R,K136A,∇137K), SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K) or SakSTAR(K35A,E65Q,K74R, D82A, S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K) in patients with peripheral arterial occlusion

Compound Patient Id.	Neutralizing antibody activity (µg/ml)		
	Before	3 weeks	4 weeks
SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R,K136A,∇137K) (SY118)			
VCL	0.2	46	50
REN	0.1	1.6	0.9
HOL	0.2	22	18
PAR	0.1	19	15
FRA	1.2	15	39
MAC	0.0	-	-
Median	0.15	19	18
SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T, K135R,K136A,∇137K) (SY141)			
VERH	0.2	0.3	0.2
DUB	0.2	4.3	2.0
VAP	0.0	0.0	0.0
WYN	0.2	10	5.6
HOR	0.2	0.1	0.0
AND	0.8	-	100
Median	0.2	0.3	1.1
SakSTAR(K35A,E65Q,K74R,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T, K135R,K136A,∇137K) (SY145)			
LIN	0.0	5.5	3.8
DEL	0.2	1.6	1.9
LAM	0.2	164	190
BAS	0.1		
TOU			
Median	0.15	5.5	3.8

Table 18: **Immunogenicity of SakSTAR variants in patients with peripheral arterial occlusion**

	n	Neutralizing activity (µg/ml)	>5 µg/ml	Specific IgG (µg/ml)	Code
SakSTAR	69	12 (4 - 100)	56	380 (81 - 1850)	-
SakSTAR(K74Q,E80A,D82A,K130T,K135R)	6	9.0 (0.1 - 23)	3	420 (31 - 730)	SY15
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	18	1.5 (0.2 - 7.0)	5	30 (24 - 100)	SY19
SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130T,K135R,K136A,∇137K)	6	27 (17 - 49)	5	2000 (1300 - 3600)	SY118
SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K	6	0.7 (0.1 – 4.3)	2	7.7 (5.1 – 510)	SY141
SakSTAR(K35A,E65Q,K74R,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K	3	4.7	1	-	SY145

Data represent median and 15-85 percentile range.

Table 19: Cysteine substitution variants of SakSTAR

Variant	Spec. Act. (kU/mg)	Dimerization level (%)	PEG derivatization	Clot lysis *in vitro* (C_{50} in µg/ml)	t1/2(α) (min)	Clp (ml/min)	Antibody Absorption (Pool 40, %)
SakSTAR	130	0	none	0.33	2.0	2.2	95
SakSTAR (K102C)	143	0	none	0.29	nd	nd	95
SakSTAR (K102C-PEG)	108	0	1	0.60	3.0	0.32	
SakSTAR (K109C) monomeric	100	0	none	0.52	nd	nd	
SakSTAR (K109C) dimeric	1,650	>60	none	0.17	3.6	0.52	90
	2,235	>95	none	0.12	nd	nd	

Table 20: **Cysteine-substitution variants of SakSTAR with reduced immunogenicity, substituted with maleimide-polyethylene glycol**

Code		Specific activity (kU/mg)	Fibrinolytic potency: Human plasma (C_{50}: µg/ml)	Fibrinolytic potency: Hamsters bolus (C_{50}: µg/kg)	Clp (ml/min)	Antibody absorption P40 (%)
-	SakSTAR	130	0.23	120	2.2	95
SY19	SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	140	0.24		3.7	57
SY19(S3C-SP5)*	SakSTAR(**S3C-SP5**,E65D,K74R,E80A,D82A,K130T,K135R)	51	0.37	42	0.45	58
SY19(S3C-MP5)*	SakSTAR(**S3C-MP5**,E65D,K74R,E80A,D82A,K130T,K135R)	20	0.65		0.28	50
SY19(S2C-SP5,S3C-SP5)	SakSTAR(**S2C-SP5,S3C-SP5**,E65D,K74R,E80A,D82A,K130T,K135R)	43	0.42	20	0.15	57
SY19(S3C-P20)	SakSTAR(**S3C-P20**,E65D,K74R,E80A,D82A,K130T,K135R)	60	0.70	18	0.065	57
SY19(S3C-P10)	SakSTAR(**S3C-P10**,E65D,K74R,E80A,D82A,K130T,K135R)	17	0.56	20	0.19	51
SY141	SakSTAR(K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K)	3,700	0.19		0.95	24
SY141(S3C-SP5)	SakSTAR(**S3C-SP5**,K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K)	1,200	0.24	12		18
SY141(S2C-SP5,S3C-SP5)	SakSTAR(**S2C-SP5,S3C-SP5**,K35A,E65Q,K74Q,D82A,S84A,T90A,E99D,T101S,E108A,K109A,K130T,K135R,K136A,∇137K)	1,400	0.28			18
SY160(S3C-P20)	SakSTAR(**S3C-P20**,K35A,E65Q,K74Q,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T,K135R)	65	0.33	6	0.08	32
SY161(S3C-MP5)	SakSTAR(**S3C-MP5**,K35A,E65Q,K74R,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T,K135R)	71	0.36	15	0.56	35
SY161(S3C-P10)	SakSTAR(**S3C-P10**,K35A,E65Q,K74R,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T,K135R)	66	0.40	9	0.15	38
SY161(S3C-P20)	SakSTAR(**S3C-P20**,K35A,E65Q,K74R,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T,K135R)	155	0.32	8	0.04	44

*SP5: OPSS-PEG 5 kDa; MP5: MAL-PEG 5 kDa; P10: MAL-PEG 10 kDa; P20: MAL-PEG 20 kDa.

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CLAIMS

1. Staphylokinase derivatives showing a reduced immunogenicity as compared to wild-type staphylokinase, after administration to patients with arterial thrombosis.

2. Staphylokinase derivatives as claimed in claim 1 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by another amino acid thus reducing the reactivity with a panel of murine monoclonal antibodies.

3. Staphylokinase derivatives as claimed in claim 1 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by another amino acid thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase.

4. Staphylokinase derivatives as claimed in claim 1 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by other amino acids, without reducing the specific activity by more than 50 percent.

5. Staphylokinase derivatives SakSTAR(K35X, G36X,E65X,K74X,E80X,D82X,K102X,E108X,K109X,K121X,K130X, K135X,K136X,+137X) having the amino acid sequence as depicted in figure 1 in which one or more of the amino acids Lys in position 35, Gly in position 36, Glu in position 65, Lys in position 74, Glu in position 80, Asp in position 82, Lys in position 102, Glu in position 108, Lys in position 109, Lys in position 121, Lys in position 130, Lys in position 135 and/or Lys in position 136 have been replaced with other amino acids and/or in which one amino acid has been added at the COOH-terminus, thus altering the immunogenicity after administration in patients, without markedly reducing the specific activity.

6. Staphylokinase derivatives listed in Tables 1, 3, 4, 5, 6, 7, 8, 13, 19 and 20, having the amino acid sequence as depicted in figure 1 in which the indicated amino acids have been replaced by other amino acids thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase, without reducing the specific activity.

7. Staphylokinase derivative as claimed in claims 1-6 selected from the group consisting of SakSTAR(K74A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E75A), SakSTAR(E80A,D82A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(S34G,G36R, H43R), SakSTAR(K35A), SakSTAR(D82A), SakSTAR(D82A,S84A), SakSTAR(T90A), SakSTAR(Y92A), SakSTAR(K130A), SakSTAR(V132A), SakSTAR(S34G,G36R,H43R), SakSTAR(G36R), SakSTAR(H43R), SakSTAR(G36R,K74R), SakSTAR(K35E), SakSTAR(K74Q), SakSTAR(K130T), SakSTAR(V132L), SakSTAR(V132T), SakSTAR(V132N), SakSTAR(V132R), SakSTAR(K130T,K135R), SakSTAR(G36R,K130T,K135R), SakSTAR(K74R,K130T,K135R), SakSTAR(K74Q,K130T,K135R), SakSTAR(G36R,K74R,K130T,K135R), SakSTAR(G36R,K74Q, K130T,K135R), SakSTAR(G36R,H43R,K74R,K130T,K135R), SakSTAR(E65A,K74Q,K130T,K135R), SakSTAR(E65Q,K74Q, K130T,K135R), SakSTAR(K74Q,K86A,K130T,K135R), SakSTAR(E65Q,T71S,K74Q,K130T,K135R), SakSTAR(K74Q, K130A,K135R), SakSTAR(E65Q,K74Q,K130A,K135R), SakSTAR(K74Q,K130E,K135R), SakSTAR(K74Q,K130E, V132R,K135R), SakSTAR(E65Q,K74Q,T90A,K130A,K135R), SakSTAR(E65Q,K74Q,N95A,K130A,K135R), SakSTAR(E65Q,K74Q, E118A,K130A,K135R), SakSTAR(E65Q,K74Q,N95A,E118A,K130A, K135R), SakSTAR(N95A,K130A,K135R), SakSTAR(E65Q,K74Q, K109A,K130,K135R), SakSTAR(E65Q,K74Q,E108A,K109A, K130T,K135R), SakSTAR(E65Q,K74Q,K121A,K130T,K135R), SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,+137K), SakSTAR(E80A,D82A,K130T,K135R), SakSTAR(K74R,E80A,D82A, K130T,K135R), SakSTAR(K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A,K74R,E80A,D82A,K130T,K135R), SakSTAR(E65D, K74R,E80A,D82A,K130T,K135R), SakSTAR(E65S,K74R,E80A,

D82A,K130T,K135R), SakSTAR(S34G,G36R,K74R,K130T,K135R), SakSTAR(E65A,K74R,E80A,D82A,K130T,K135R), SakSTAR(E65N, K74R,E80A,D82A,K130T,K135R), SakSTAR(E65Q,K74R,E80A, D82A,K130T,K135R), SakSTAR(K57A,E58A,E61A,E80A,D82A, K130T,K135R), SakSTAR(E65D,K74Q,E80A,D82A,K130T,K135R), SakSTAR(E65Q,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A, E65D,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K74R,E80A,D82A, S103A,K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K109A, K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K130T, K135R,K136A), SakSTAR(E65Q,K74Q,D82A,S84A,K130T,K135R), SakSTAR(K35A,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A, E65D,K74R,E80A,D82A,K130T,K135R).

8. SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) having the code SY19.

9. SakSTAR(K35A,E65Q,K74R,E80A,D82A,T90A,E99D, T101S,E108A,K109A,K130T,K135R) having the code SY161.

10. Staphylokinase derivatives as claimed in claims 1-9 having an amino acid substituted with Cys, resulting in dimerization and/or increased specific activity and/or reduced clearance and/or increased thrombolytic potency.

11. Staphylokinase derivatives as claimed in claims 1-10 with polyethylene glycol substitution, characterized by a maintained specific activity and a significantly reduced plasma clearance.

12. Staphylokinase derivatives as claimed in claim 10 wherein the Cys is chemically modified with polyethylene glycol with molecular weights up to 20 kDa.

13. Staphylokinase derivatives as claimed in claim 12 wherein selected amino acids in the NH_2-terminal region of 10 amino acids, are substituted with Cys, which is chemically modified with polyethylene glycol with molecular weights up to 20 kDa, which derivatives are characterized by a significantly reduced plasma clearance and maintained thrombolytic potency upon single intravenous bolus administration at a reduced dose.

14. Staphylokinase derivative as claimed in claim 13, wherein the serine in position 2 or 3 is

substituted with a cystein and the cystein is chemically modified with polyethylene glycol having a molecular weight of 5, 10 or 20 kDa.

15. Staphylokinase derivative as claimed in claim 14, which derivative is SY161(S3C-MP5) as defined in table 20.

16. Staphylokinase derivative as claimed in claim 14, which derivative is SY161(S3C-P10) as defined in table 20.

17. Staphylokinase derivative as claimed in claim 14, which derivative is SY161(S3C-P20) as defined in table 20.

18. Staphylokinase derivative as claimed in claim 14, which derivative is SY19(S3C-MP5) as defined in table 20.

19. Staphylokinase derivative as claimed in claim 14, which derivative is SY19(S3C-SP5) as defined in table 20.

20. Staphylokinase derivative as claimed in claim 14, which derivative is SY19(S2C-SP5,S3C-SP5) as defined in table 20.

21. Staphylokinase derivative as claimed in claim 14, which derivative is SY19(S3C-P20) as defined in table 20.

22. Staphylokinase derivative as claimed in claim 14, which derivative is SY19(S3C-P10) as defined in table 20.

23. Dimer of two staphylokinase derivatives as claimed in claim 10.

24. Method for producing the staphylokinase derivatives as claimed in claims 1 to 10, comprising the steps of:

a. preparing a DNA fragment comprising at least the part of the coding sequence of staphylokinase that provides for its biological activity;

b. performing in vitro site-directed mutagenesis on the DNA fragment to replace one r more

codons for wild-typ amino acids by a codon for another amino acid;

c. cloning the mutated DNA fragment in a suitable vector;

d. transforming or transfecting a suitable host cell with the vector; and

e. culturing the host cell under conditions suitable for expressing the DNA fragment.

25. Method as claimed in claim 24, wherein the DNA fragment is a 453 bp EcoRI-HindIII fragment of the plasmid pMEX602sakB, the in vitro site-directed mutagenesis is performed and the mutated DNA fragment is expressed in E. coli.

26. Pharmaceutical composition comprising at least one of the staphylokinase derivatives as claimed in claims 1 to 23 together with a suitable excipient.

27. Pharmaceutical composition as claimed in claim 26 for treating arterial thrombosis.

1 14
Ser Ser Ser Phe Asp Lys Gly Lys Tyr Lys Lys Gly Asp Asp

15 28
Ala Ser Tyr Phe Glu Pro Thr Gly Pro Tyr Leu Met Val Asn

29 42
Val Thr Gly Val Asp Ser Lys Gly Asn Glu Leu Leu Ser Pro

43 56
His Tyr Val Glu Phe Pro Ile Lys Pro Gly Thr Thr Leu Thr

57 70
Lys Glu Lys Ile Glu Tyr Tyr Val Glu Trp Ala Leu Asp Ala

FIG. 1-I

71 84
Thr Ala Tyr Lys Glu Phe Arg Val Val Glu Leu Asp Pro Ser

85 98
Ala Lys Ile Glu Val Thr Tyr Tyr Asp Lys Asn Lys Lys Lys

99 112
Glu Glu Thr Lys Ser Phe Pro Ile Thr Glu Lys Gly Phe Val

113 126
Val Pro Asp Leu Ser Glu His Ile Lys Asn Pro Gly Phe Asn

127 136
Leu Ile Thr Lys Val Val Ile Glu Lys Lys

FIG. 1-II

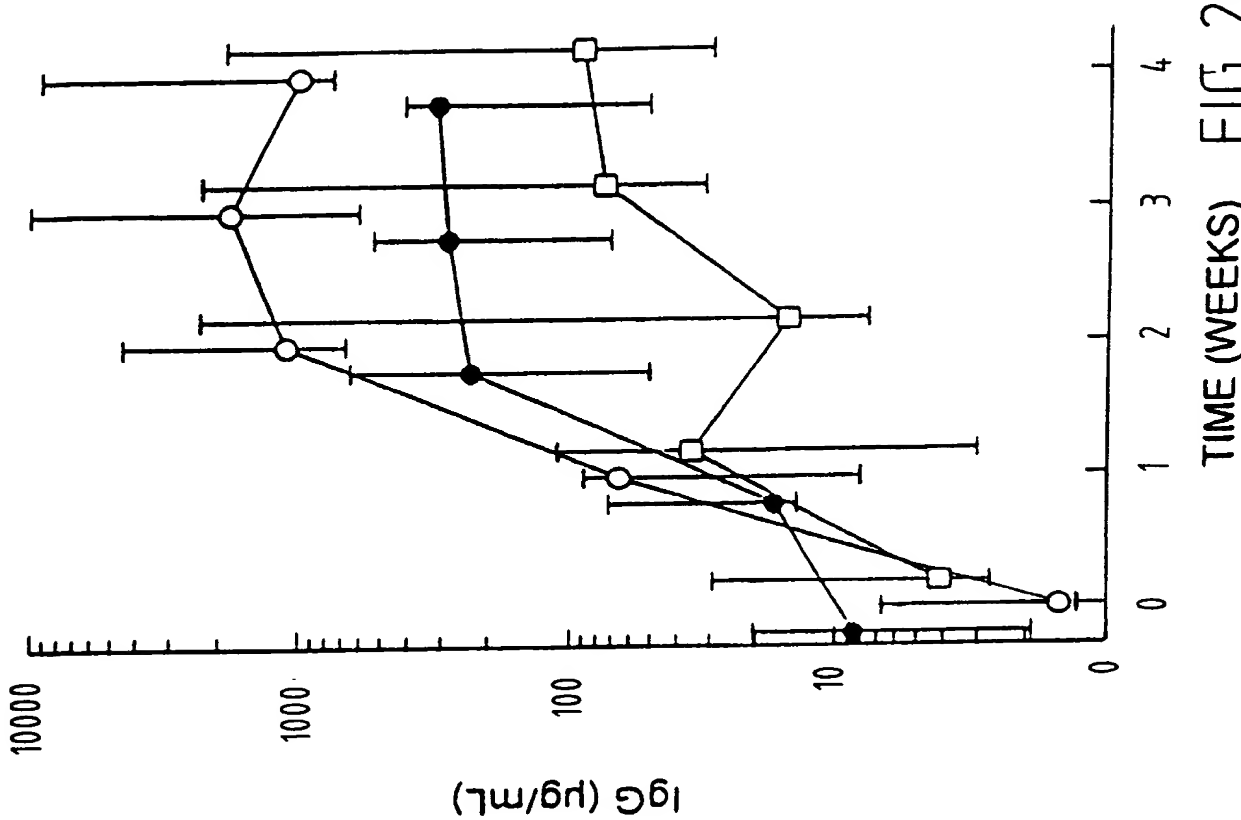

IgG (µg/mL)
10000
1000
100
10
0
0
1
2
3
4
TIME (WEEKS)

FIG. 2B

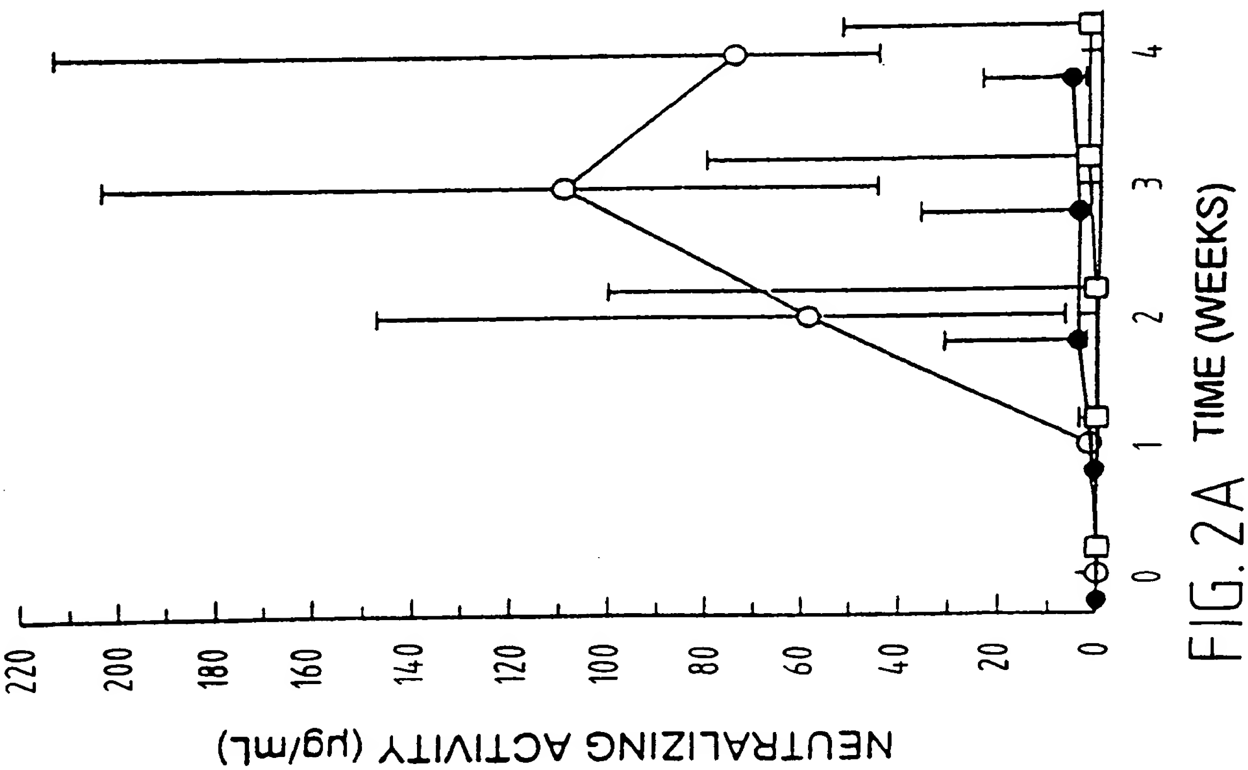

NEUTRALIZING ACTIVITY (µg/mL)
220
200
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
TIME (WEEKS)

FIG. 2A

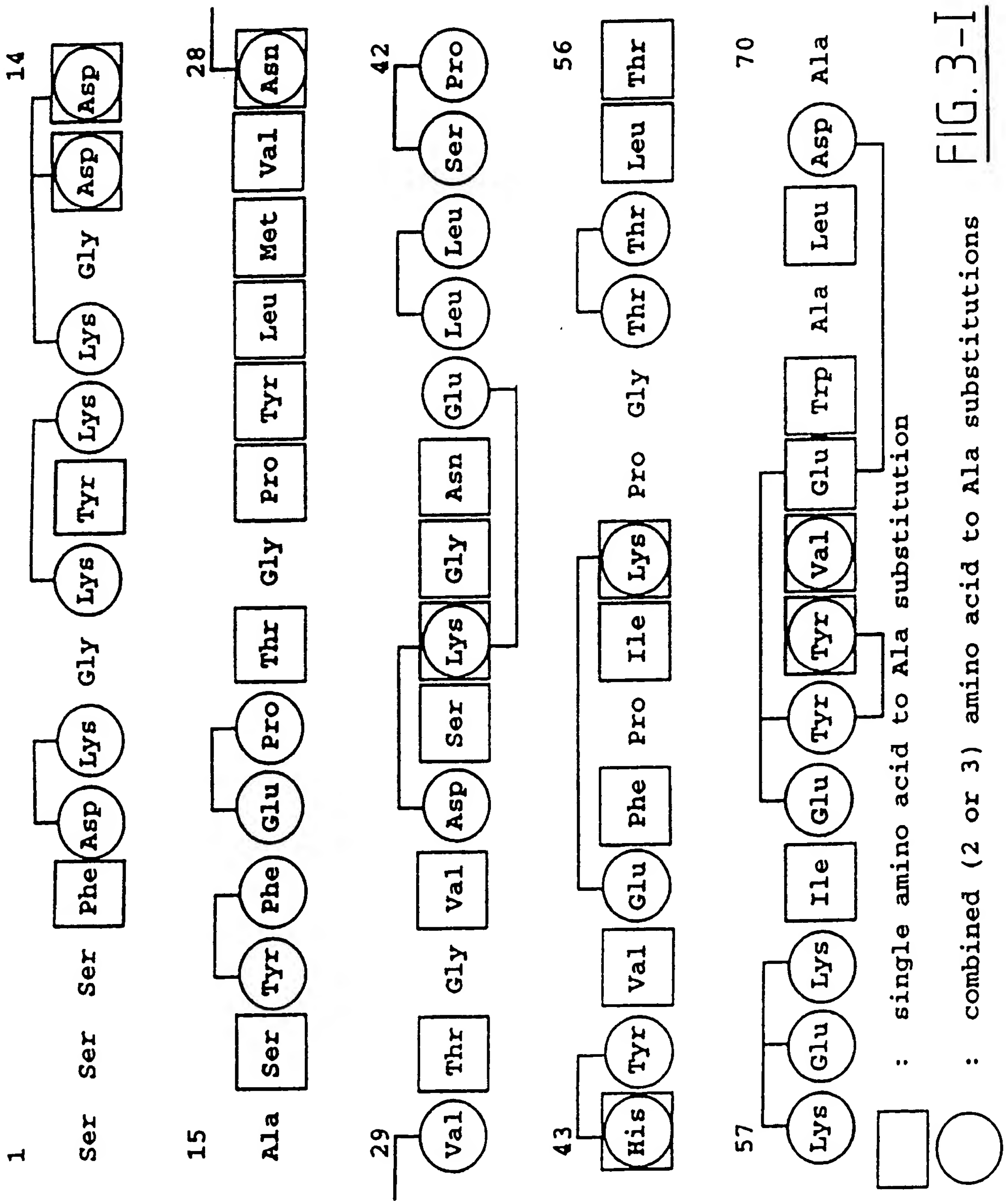

1 Ser Ser Ser Phe Asp Lys Gly Lys Tyr Lys Lys Gly Asp Asp 14
15 Ala Ser Tyr Phe Glu Pro Thr Gly Pro Tyr Leu Met Val Asn 28
29 Val Thr Gly Val Asp Ser Lys Gly Asn Glu Leu Leu Ser Pro 42
43 His Tyr Val Glu Phe Pro Ile Lys Pro Gly Thr Thr Leu Thr 56
57 Lys Glu Lys Ile Glu Tyr Tyr Val Glu Trp Ala Leu Asp Ala 70
: single amino acid to Ala substitution
: combined (2 or 3) amino acid to Ala substitutions

FIG. 3-I

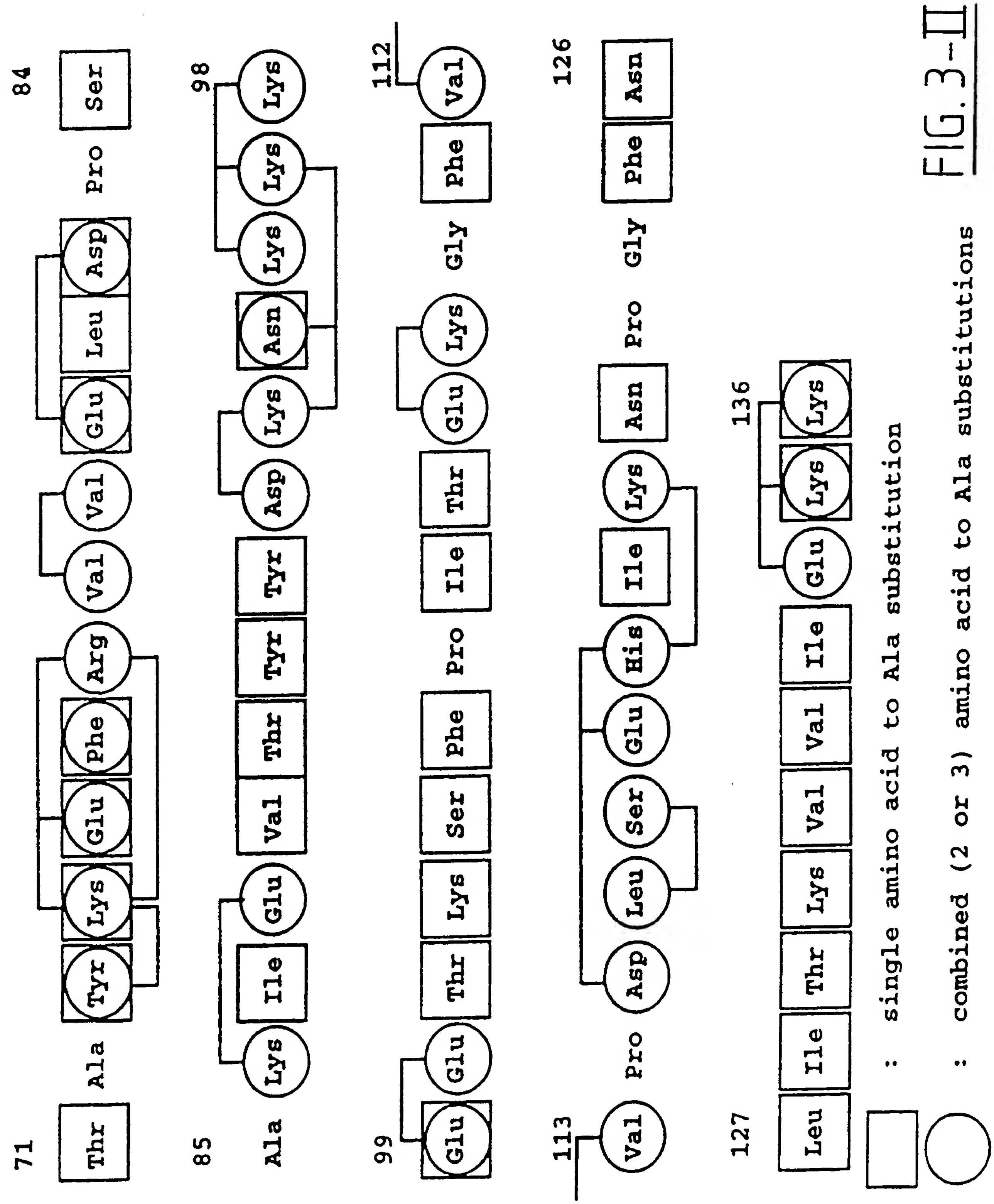

71 Thr Ala Tyr Lys Glu Phe Arg Val Val Glu Leu Asp Pro Ser 84
85 Ala Lys Ile Glu Val Thr Tyr Tyr Asp Lys Asn Lys Lys Lys 98
99 Glu Glu Thr Lys Ser Phe Pro Ile Thr Glu Lys Gly Phe Val 112
113 Val Pro Asp Leu Ser Glu His Ile Lys Asn Pro Gly Phe Asn 126
127 Leu Ile Thr Lys Val Val Ile Glu Lys Lys 136
: single amino acid to Ala substitution
: combined (2 or 3) amino acid to Ala substitutions

FIG. 3-II

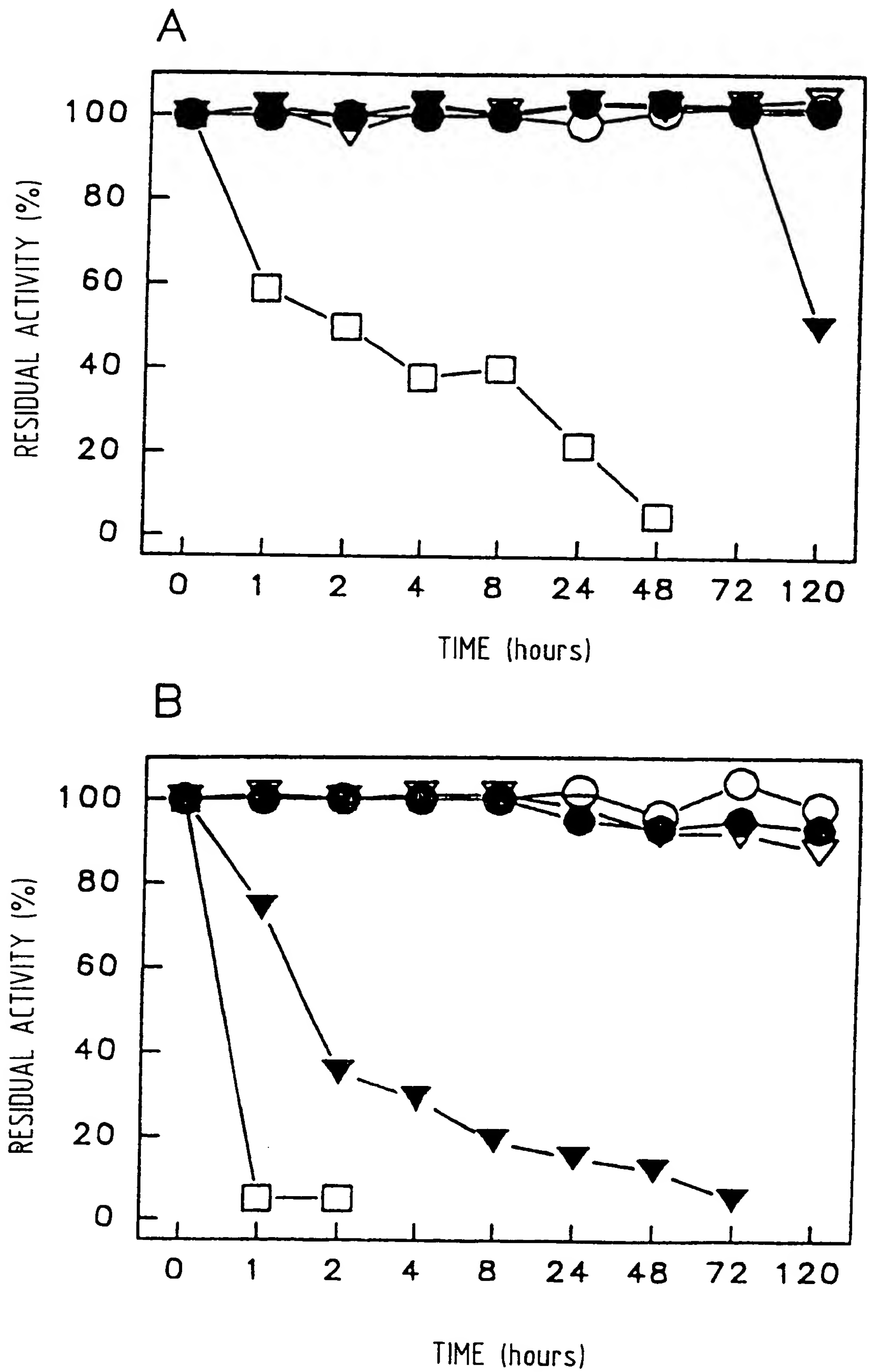
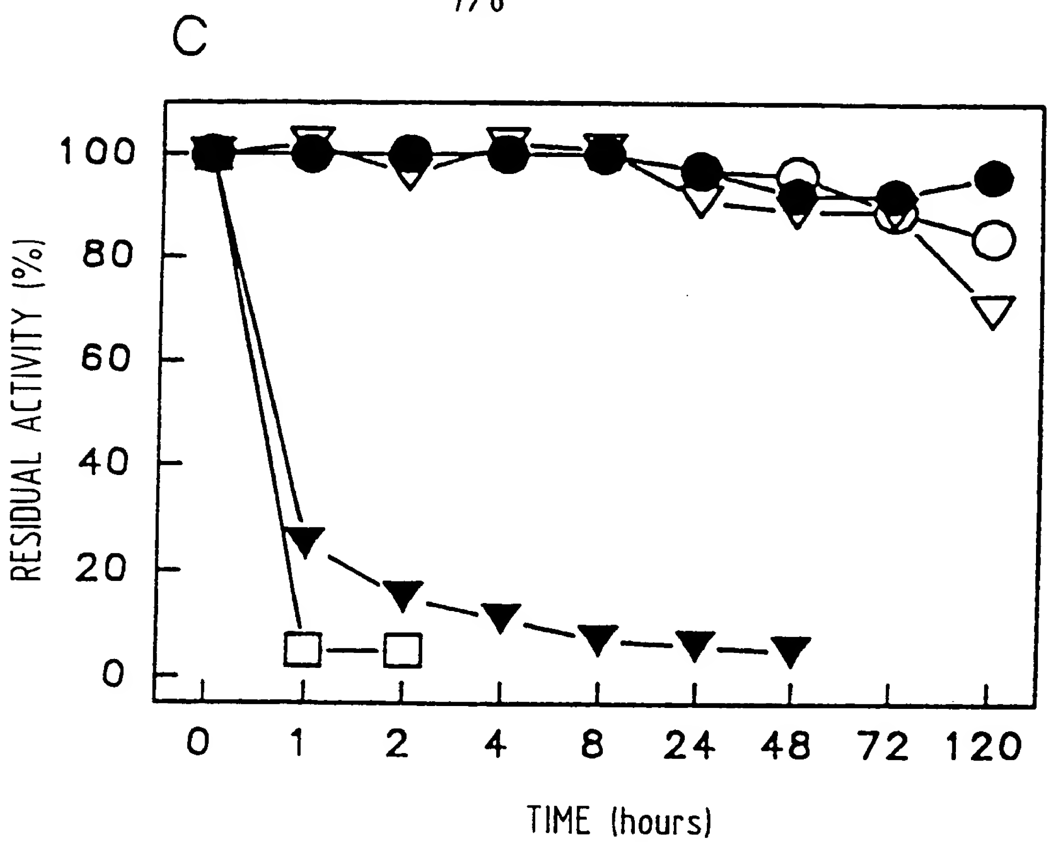

A
RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
0
1
2
4
8
24
48
72
120
TIME (hours)
B
RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
0
1
2
4
8
24
48
72
120
TIME (hours)

FIG. 4-I


C
100
80
60
40
20
0
RESIDUAL ACTIVITY (%)
0
1
2
4
8
24
48
72
120
TIME (hours)

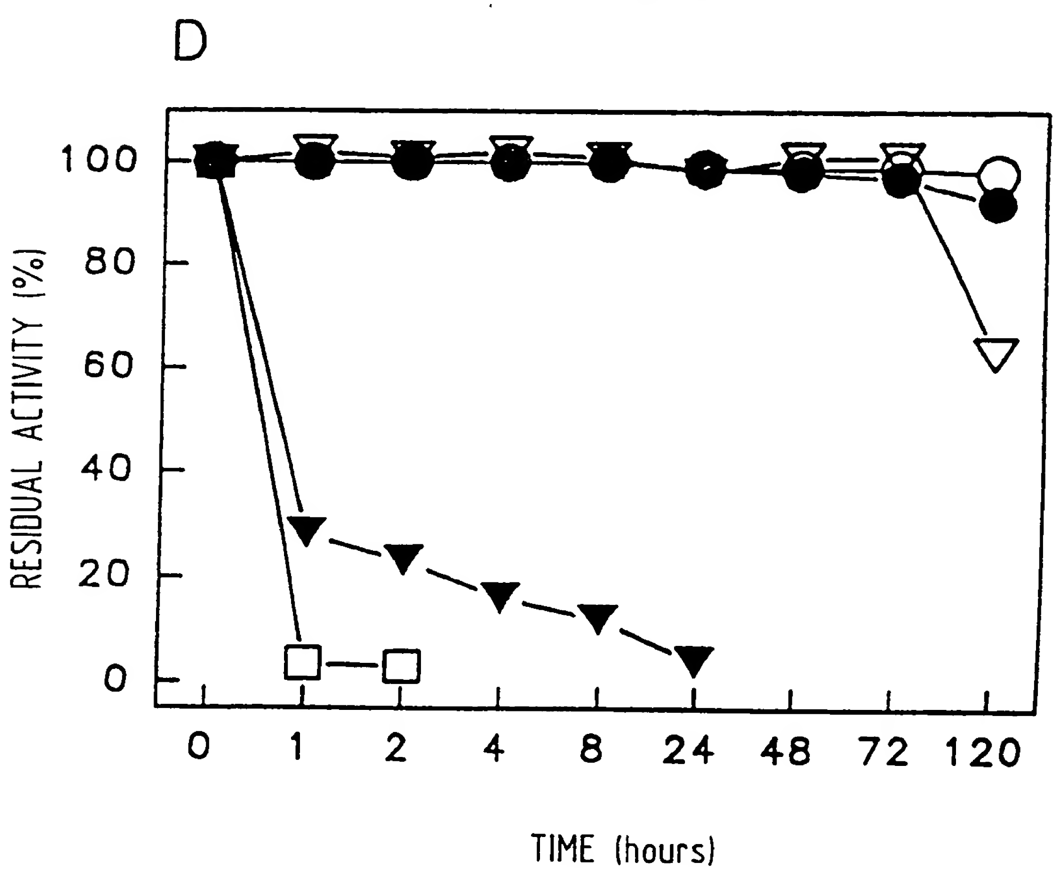

D
100
80
60
40
20
0
RESIDUAL ACTIVITY (%)
0
1
2
4
8
24
48
72
120
TIME (hours)

FIG. 4-II

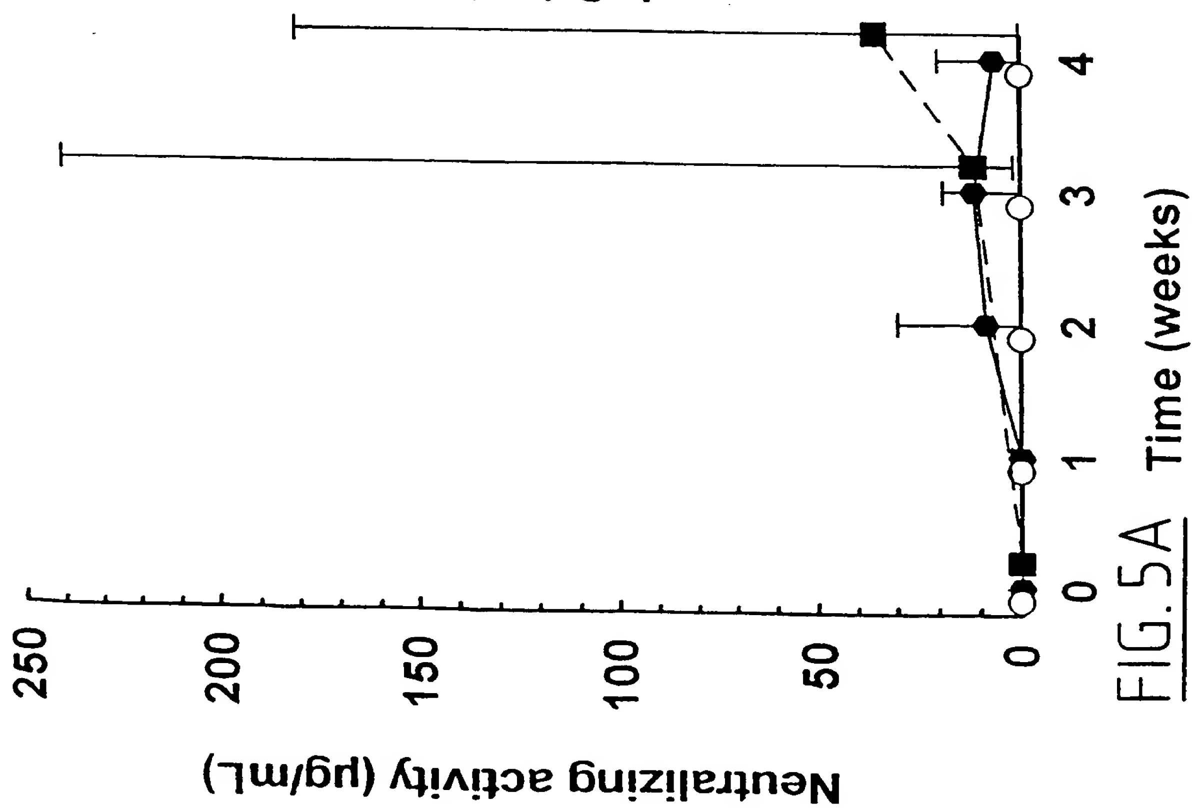

Neutralizing activity (µg/mL)
250
200
150
100
50
0
0
1
2
3
4
Time (weeks)

FIG. 5A

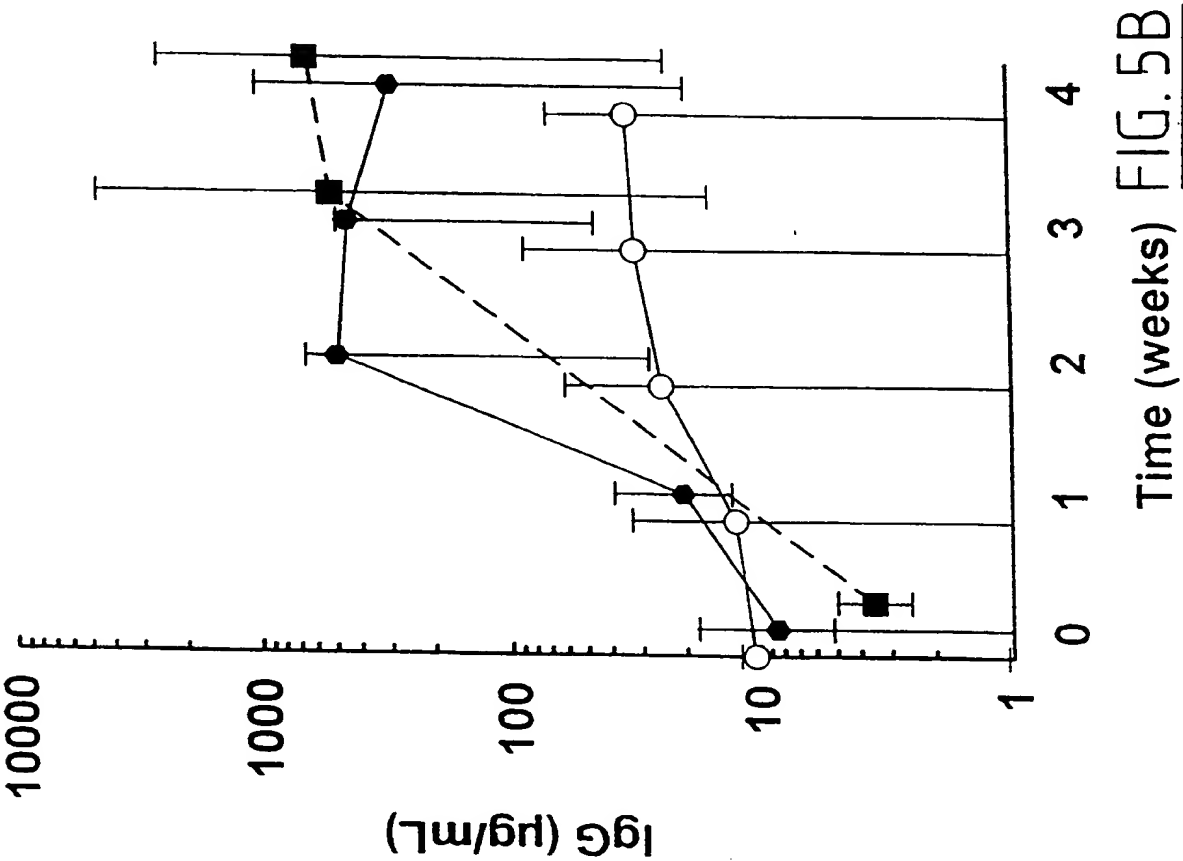

IgG (µg/mL)
10000
1000
100
10
1
0
1
2
3
4
Time (weeks)

FIG. 5B

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PATENT COOPERATION TREATY

REC'D 30 MAY 2000
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference L/UV24/102	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP99/00748	International filing date *(day/month/year)* 04/02/1999	Priority date *(day/month/year)* 04/02/1998
International Patent Classification (IPC) or national classification and IPC C12N15/31		
Applicant LEUVEN RESEARCH & DEVELOPMENT VZW et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☒ Priority
   III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☒ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand 02/09/1999	Date of completion of this report 26.05.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Morawetz, R Telephone No. +49 89 2399 8155

I. Basis of the r port

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-86 as originally filed

Claims, No.:

1-30 as received on 01/05/2000 with letter of 01/05/2000

Drawings, sheets:

1/5-5/5 as originally filed

2. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

   see separate sheet

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has

been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 1-5, 9-13, 26 and in part 6, 14-17, 27-30.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 1-5, 9-13, 26 and in part 6, 14-17, 27-30.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	6 (part), 7, 8, 14 (part),15-17 (part), 18-25
	No:	Claims	14 (part), 27- 30
Inventive step (IS)	Yes:	Claims	6 (part*), 14 (part*), 7, 8, 15 (part*) -17 (part*), 18-25
	No:	Claims	6 (part'), 14 (part'), 15 (part') -17 (part')
Industrial applicability (IA)	Yes:	Claims	6 (part), 7, 8, 14 (part)-17 (part), 18-25, 27 (part)-30 (part)
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

s e s parat she t

Re Item I

Basis

1. The amendments filed with the letter dated 01.05.2000 appear to be allowable under Article 34(2)(b) PCT. The amendments concerned are the following:

1.1. Claim 6 corresponds to original claim 7 of which not novel SakSTAR variants have been omitted.
Claims 7 and 8 correspond to original claims 8 and 9, respectively.
Claim 14 corresponds to original claim 7.
Claims 15-25 correspond to original claims 12-22, respectively. In claims 18-25 the reference to table 20 has been substituted by the SakSTAR indication corresponding to the code used in Table 20.
Claims 27-30 correspond to original claims 24-27, respectively.

2. The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established (claims 1-5, 9-13, 26 and in part 6, 14-17, 27-30) need not be the subject of international preliminary examination (Rule 66.1 (e) PCT).

   Amendments of these claims are thus of no consequence for the establishment of this report.

   Applicant's attention is however drawn to the fact that introduction of the feature "provided that the other amino acid is not alanine" into claims 1-3 and of the feature "provided that at least one amino acid is replaced with an amino acid other than alanine" into claim 5 is considered to introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT.

Re Item II

Priority

1. D1 (COLLEN, D. ET AL., FIBRINOLYSIS & PROTEOLYSIS, (JUNE, 1998) VOL. 12, NO. SUPPL. 1, PP. 30. MEETING INFO.: XIVTH INTERNATIONAL CONGRESS ON FIBRINOLYSIS AND THROMBOLYSIS LJUBLJANA, SLOVENIA

JUNE 22-26, 1998), which discloses several of the presently claimed SakSTAR variants is not considered part of the prior art (Rule 64 PCT) as the relevant subject-matter of present application can validly claim the priority of EP application 98200323.8 dated 04.02.1998 (see Table 10 and Example 8).

Re Item III

Non-establishment of report with regard to novelty, inventive step or industrial applicability

1. No report will be established for claims relating to inventions in respect of which no international search report has been established (Article 34(4)(a) and Rule 66.1 (e) PCT).

Re Item IV

Lack of unity of invention

1. Rule 13 PCT stipulates that the international application shall relate to one invention only or to a group so linked as to form a single general inventive concept. Where a group of inventions is claimed in one and the same international application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding "special technical features", i.e. technical features that define a novel and inventive contribution over the prior art.

2. Reference is made to the following documents, the numbering corresponds to the listing of the documents in the international search report:

D2: COLLEN D ET AL., CIRCULATION, (1997 JAN 21), 95 (2): 455-62
D3: COLLEN D ET AL., CIRCULATION, (1996 JUL 15) 94 (2): 207-16
D4: COLLEN D ET AL., CIRCULATION, (1996 JUL 15) 94 (2): 197-206
D5: COLLEN D ET AL., CIRCULATION, (1997 JAN 21) 95 (2): 463-72
D6: EP-A-0 721 982 (1996-07-17)

The document D7 was cited in the application (page 86, line 10-13):

D7: INADA Y ET AL., TIBTECH 13: 86-91, (1995).

3. The only "special technical feature" common to all present claims is that they are concerned with staphylokinase derivatives having reduced immunogenicity and thrombolytic efficacy. However, staphylokinase derivatives having reduced immunogenicity and thrombolytic efficacy in general and several of the presently claimed derivatives in particular are well known in the prior art (see D2-D6). Consequently, this common feature does not unitarily link the present set of claims and in the absence of another special technical feature, the present set of claims lacks an unifying concept and each staphylokinase derivative is considered as a separate invention.

4. Although all claimed inventions have been the subject of examination, the objection regarding lack of unity may be pursued at a later time point, e.g. in the regional phase of the application.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The present application does not satisfy the criterion set forth in Article 33(2) PCT because the subject-matter of claims 14 (part), 27- 30 is not new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).

1.1. The subject-matter of claim 14(part), insofar directed to the following staphylokinase derivatives is anticipated by the prior art.

SakSTAR(K74A, E75A,R77A) has been disclosed in D2, D3, D4, D5 and D6.
SakSTAR(K35A, E75A) has been disclosed in D2.
SakSTAR(E75A) has been disclosed in D2 and D5.
SakSTAR(E80A, D82A) has been disclosed in D2, D3, D4 and D6.
SakSTAR(E80A) has been disclosed in D2.

SakSTAR(D82A) has been disclosed in D2.
SakSTAR(E75A, D82A) has been disclosed in D2.

1.2. The subject-matter of claims 27-30 is anticipated by the prior art.

The method of claim 27 is disclosed in D4 (page 199, right hand column, 2nd paragraph - page 202, left hand column, 1st paragraph), D6 (page 3, line 47-51).

The method of claim 28 is disclosed in D4 (page 199, right hand column, 2nd paragraph - page 202, left hand column, 1st paragraph).

The pharmaceutical compositions of claims 29 and 30 are anticipated by D3 (whole document), D5 (whole document) and D6 (page 3, line 55-59).

1.3. The subject-matter of claim 14 (part), insofar directed to staphylokinase derivatives other than the ones indicated in V.1.1. above and the subject-matter of claims 6 (part), 7, 8, 15-17 (part), 18-25, appears to be new in view of the available prior art.

2. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of claims 6 (part'), 14 (part'), 15 (part') -17 (part') does not involve an inventive step as defined in the regulations (Rule 65 (1)-(2) PCT).

2.1. The subject-matter of claims 6 (part') and 14 (part'), insofar directed to SakSTAR(K35A), SakSTAR(K130A) and SakSTAR(V132A) lacks an inventive step.

D4 discloses (Figure 1) the amino acid sequence of SakSTAR with indication of the charged amino acid clusters that are substituted with alanine in order to evaluate the immunoreactivity of site-specific mutants. Several of these "charged cluster-to-alanine" substitutions variants induce less antibody formation in patients than wild-type recombinant staphylokinase (SakSTAR), but their specific activities are reduced by 50%. D4 concludes that it is possible to produce engineered variants of staphylokinase that are functional but less antigenic than the wild-type molecule.

D2 and D5 study the effect of the reversal of one or more of these "charged cluster-to-alanine" substituted amino acids to the wild type residues on the ratio of activity to antigenicity.

The subject-matter of claims 6 (part') and 14 (part'), insofar directed to further alanine-substitution mutants of staphylokinase is, thus, considered obvious in view of D4 in combination with D2 or D5.

2.2. Claims 15 (part') -17 (part') concern embodiments which are familiar to the skilled person (see D2-D7). Consequently, they would only be considered inventive if they were based upon a new and inventive staphylokinase derivative. For the present claims 15 (part') -17 (part'), insofar related to the non-novel or non-inventive subject-matter of claims 6 (part') and 14 (part') this is not the case. Therefore the subject-matter of these claims is considered to be obvious.

3. The present application satisfies the criterion set forth in Article 33(3) PCT because the subject-matter of claims 6 (part*), 14 (part*), 7, 8, 15 (part*) -17 (part*), 18-25 involves an inventive step as defined in the regulations (Rule 65 (1)-(2) PCT).

3.1. Claims 6 (part*) and 14 (part*), insofar directed to further substitution mutants of staphylokinase in which amino acids were substituted with amino acids other than Ala and insofar directed to combination variants of SakSTAR(K130T, K135T) and SakSTAR(E80A, D82A, K130T, K135T) are considered to meet the requirements of the PCT with respect to inventive step.

Document D5, which is considered to represent the most relevant state of the art, discloses (whole document) staphylokinase derivatives from which the subject-matter of claims 6 (part*) and 14 (part*) differs in that it relates to staphylokinase derivatives having different amino acid substitutions.
The problem to be solved by the present invention may therefore be regarded as the provision of alternative staphylokinase derivatives having reduced antibody induction but intact thrombolytic potency.

The solution to this problem proposed in claims 6 (part*) and 14 (part*) of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:

The prior art discloses staphylokinase derivatives having reduced antibody induction but intact thrombolytic potency due to "charged cluster-to-alanine" substitutions and reversal of one or more of these substituted amino acids to the wild-type residues (see e.g. D5).

The prior art neither discloses nor suggests that staphylokinase derivatives which have substitutions other than alanine would still show reduced antibody induction while retaining intact thrombolytic potency.

3.2. Claims 7, 8 and 18-25 which relate to further combination variants of SakSTAR(K130T, K135T) and cysteine-substitutions variants of SakSTAR with reduced immunogenicity, substituted with maleimide-polyethylene glycol are also considered to meet the requirements of the PCT with respect to inventive step.

3.3. Claims 15 (part*) -17 (part*) insofar related to the novel and inventive subject-matter of claims 6 (part*), 7, 8 and 14 (part*) are considered to meet the requirements of the PCT with respect to inventive step.

<u>Re Item VIII</u>

Certain observations on the international application

1. Claim 15 has been interpreted as referring to claim 14 and not to claim 9.

CLAIMS

1. Staphylokinase derivatives having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by another amino acid thus reducing the reactivity with a panel of murine monoclonal antibodies provided that the other amino acid is not alanine.

2. Staphylokinase derivatives as claimed in claim 1 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by another amino acid thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase provided that the other amino acid is not alanine.

3. Staphylokinase derivatives as claimed in claim 1 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by other amino acids, without reducing the specific activity by more than 50 percent provided that the other amino acid is not alanine.

4. Staphylokinase derivatives SakSTAR(K35X, G36X,E65X,K74X,E80X,D82X,K102X,E108X,K109X,K121X,K130X, K135X,K136X,+137X) having the amino acid sequence as depicted in figure 1 in which one or more of the amino acids Lys in position 35, Gly in position 36, Glu in position 65, Lys in position 74, Glu in position 80, Asp in position 82, Lys in position 102, Glu in position 108, Lys in position 109, Lys in position 121, Lys in position 130, Lys in position 135 and/or Lys in position 136 have been replaced with other amino acids provided that the other amino acid is not alanine and/or in which one amino acid has been added at the COOH-terminus, thus altering the immunogenicity after administration in patients, without markedly reducing the specific activity.

5. Staphylokinase derivatives listed in Tables 1, 3, 4, 5, 6, 7, 8, 13, 19 and 20, having the amino acid sequence as depicted in figure 1 in which the indicated

amino acids have been replaced by other amino acids thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase, without reducing the specific activity, provided that at least one amino acid is replaced with an amino acid other than alanine.

6. Staphylokinase derivative as claimed in claims 1-5 selected from the group consisting of SakSTAR(S34G,G36R,H43R), SakSTAR(S34G,G36R,H43R), SakSTAR(G36R), SakSTAR(H43R), SakSTAR(G36R,K74R), SakSTAR(K35E), SakSTAR(K74Q), SakSTAR(K130T), SakSTAR(V132L), SakSTAR(V132T), SakSTAR(V132N), SakSTAR(V132R), SakSTAR(K130T,K135R), SakSTAR(G36R, K130T,K135R), SakSTAR(K74R,K130T,K135R), SakSTAR(K74Q, K130T,K135R), SakSTAR(G36R,K74R,K130T,K135R), SakSTAR(G36R,K74Q,K130T,K135R), SakSTAR(G36R,H43R,K74R, K130T,K135R), SakSTAR(E65A,K74Q,K130T,K135R), SakSTAR(E65Q,K74Q,K130T,K135R), SakSTAR(K74Q,K86A,K130T, K135R), SakSTAR(E65Q,T71S,K74Q,K130T,K135R), SakSTAR(K74Q,K130A,K135R), SakSTAR(E65Q,K74Q,K130A, K135R), SakSTAR(K74Q,K130E,K135R), SakSTAR(K74Q,K130E, V132R,K135R), SakSTAR(E65Q,K74Q,T90A,K130A,K135R), SakSTAR(E65Q,K74Q,N95A,K130A,K135R), SakSTAR(E65Q,K74Q, E118A,K130A,K135R), SakSTAR(E65Q,K74Q,N95A,E118A,K130A, K135R), SakSTAR(N95A,K130A,K135R), SakSTAR(E65Q,K74Q, K109A,K130,K135R), SakSTAR(E65Q,K74Q,E108A,K109A, K130T,K135R), SakSTAR(E65Q,K74Q,K121A,K130T,K135R), SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,+137K), SakSTAR(E80A,D82A,K130T,K135R), SakSTAR(K74R,E80A,D82A, K130T,K135R), SakSTAR(K74Q,E80A,D82A,K130T,K135R), Sak-STAR(K35A,K74R,E80A,D82A,K130T,K135R), SakSTAR(E65D, K74R,E80A,D82A,K130T,K135R), SakSTAR(E65S,K74R,E80A, D82A,K130T,K135R), SakSTAR(S34G,G36R,K74R,K130T,K135R), SakSTAR(E65A,K74R,E80A,D82A,K130T,K135R), SakSTAR(E65N, K74R,E80A,D82A,K130T,K135R), SakSTAR(E65Q,K74R,E80A, D82A,K130T,K135R), SakSTAR(K57A,E58A,E61A,E80A,D82A, K130T,K135R), SakSTAR(E65D,K74Q,E80A,D82A,K130T,K135R), SakSTAR(E65Q,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A,

E65D,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K74R,E80A,D82A, S103A,K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K109A, K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K130T, K135R,K136A), SakSTAR(E65Q,K74Q,D82A,S84A,K130T,K135R), SakSTAR(K35A,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A, E65D,K74R,E80A,D82A,K130T,K135R).

7. SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) having the code SY19.

8. SakSTAR(K35A,E65Q,K74R,E80A,D82A,T90A,E99D, T101S,E108A,K109A,K130T,K135R) having the code SY161.

9. Staphylokinase derivatives having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by another amino acid thus reducing the reactivity with a panel of murine monoclonal antibodies and having in addition either one or both of the following:

- at least one amino acid substituted with Cys, resulting in dimerization and/or increased specific activity and/or reduced clearance and/or increased thrombolytic potency; and/or

- polyethylene glycol substitution, resulting in a significantly reduced plasma clearance while maintaining specific activity.

10. Staphylokinase derivatives as claimed in claim 9 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by another amino acid thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase.

11. Staphylokinase derivatives as claimed in claim 9 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by other amino acids, without reducing the specific activity by more than 50 percent.

12. Staphylokinase derivatives as claimed in claim 9, named SakSTAR(K35X,G36X,E65X,K74X,E80X,D82X,K1-02X,E108X,K109X,K121X,K130X,K135X,K136X,+137X) and having the amino acid sequence as depicted in figure 1 in which

one or more of the amino acids Lys in position 35, Gly in position 36, Glu in position 65, Lys in position 74, Glu in position 80, Asp in position 82, Lys in position 102, Glu in position 108, Lys in position 109, Lys in position 121, Lys in position 130, Lys in position 135 and/or Lys in position 136 have been replaced with other amino acids and/or in which one amino acid has been added at the COOH-terminus, thus altering the immunogenicity after administration in patients, without markedly reducing the specific activity.

13. Staphylokinase derivatives as claimed in claim 9 and listed in Tables 1, 3, 4, 5, 6, 7, 8, 13, 19 and 20, having the amino acid sequence as depicted in figure 1 in which the indicated amino acids have been replaced by other amino acids thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase, without reducing the specific activity.

14. Staphylokinase derivative as claimed in claims 9-13 selected from the group consisting of SakSTAR(K74A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E75A), SakSTAR(E80A,D82A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(S34G,G36R, H43R), SakSTAR(K35A), SakSTAR(D82A), SakSTAR(D82A,S84A), SakSTAR(T90A), SakSTAR(Y92A), SakSTAR(K130A), SakSTAR(V132A), SakSTAR(S34G,G36R,H43R), SakSTAR(G36R), SakSTAR(H43R), SakSTAR(G36R,K74R), SakSTAR(K35E), SakSTAR(K74Q), SakSTAR(K130T), SakSTAR(V132L), SakSTAR(V132T), SakSTAR(V132N), SakSTAR(V132R), SakSTAR(K130T,K135R), SakSTAR(G36R,K130T,K135R), SakSTAR(K74R,K130T,K135R), SakSTAR(K74Q,K130T,K135R), SakSTAR(G36R,K74R,K130T,K135R), SakSTAR(G36R,K74Q, K130T,K135R), SakSTAR(G36R,H43R,K74R,K130T,K135R), SakSTAR(E65A,K74Q,K130T,K135R), SakSTAR(E65Q,K74Q, K130T,K135R), SakSTAR(K74Q,K86A,K130T,K135R), SakSTAR(E65Q,T71S,K74Q,K130T,K135R), SakSTAR(K74Q, K130A,K135R), SakSTAR(E65Q,K74Q,K130A,K135R), SakSTAR(K74Q,K130E,K135R), SakSTAR(K74Q,K130E,

V132R,K135R), SakSTAR(E65Q,K74Q,T90A,K130A,K135R), SakSTAR(E65Q,K74Q,N95A,K130A,K135R), SakSTAR(E65Q,K74Q, E118A,K130A,K135R), SakSTAR(E65Q,K74Q,N95A,E118A,K130A, K135R), SakSTAR(N95A,K130A,K135R), SakSTAR(E65Q,K74Q, K109A,K130,K135R), SakSTAR(E65Q,K74Q,E108A,K109A, K130T,K135R), SakSTAR(E65Q,K74Q,K121A,K130T,K135R), SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,+137K), SakSTAR(E80A,D82A,K130T,K135R), SakSTAR(K74R,E80A,D82A, K130T,K135R), SakSTAR(K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A,K74R,E80A,D82A,K130T,K135R), SakSTAR(E65D, K74R,E80A,D82A,K130T,K135R), SakSTAR(E65S,K74R,E80A, D82A,K130T,K135R), SakSTAR(S34G,G36R,K74R,K130T,K135R), SakSTAR(E65A,K74R,E80A,D82A,K130T,K135R), SakSTAR(E65N, K74R,E80A,D82A,K130T,K135R), SakSTAR(E65Q,K74R,E80A, D82A,K130T,K135R), SakSTAR(K57A,E58A,E61A,E80A,D82A, K130T,K135R), SakSTAR(E65D,K74Q,E80A,D82A,K130T,K135R), SakSTAR(E65Q,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A, E65D,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K74R,E80A,D82A, S103A,K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K109A, K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K130T, K135R,K136A), SakSTAR(E65Q,K74Q,D82A,S84A,K130T,K135R), SakSTAR(K35A,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A, E65D,K74R,E80A,D82A,K130T,K135R).

15. Staphylokinase derivatives as claimed in claim 9 wherein the Cys is chemically modified with polyethylene glycol with molecular weights up to 20 kDa.

16. Staphylokinase derivatives as claimed in claim 15 wherein selected amino acids in the NH_2-terminal region of 10 amino acids, are substituted with Cys, which is chemically modified with polyethylene glycol with molecular weights up to 20 kDa, which derivatives are characterized by a significantly reduced plasma clearance and maintained thrombolytic potency upon single intravenous bolus administration at a reduced dose.

17. Staphylokinase derivative as claimed in claim 16, wherein the serine in position 2 or 3 is substituted with a cystein and the cystein is chemically

modified with polyethylene glycol having a molecular weight of 5, 10 or 20 kDa.

18. Staphylokinase derivative as claimed in claim 17, which derivative is SakSTAR(S3C-MP5,K35A,E65Q, K74R,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T,K135R) (SY161(S3C-MP5)).

19. Staphylokinase derivative as claimed in claim 17, which derivative is SakSTAR(S3C-P10,K35A, E65Q,K74R,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T, K135R) (SY161(S3C-P10)).

20. Staphylokinase derivative as claimed in claim 17, which derivative is SakSTAR(S3C-P20,K35A, E65Q,K74R,E80A,D82A,T90A,E99D,T101S,E108A,K109A,K130T, K135R) (SY161(S3C-P20)).

21. Staphylokinase derivative as claimed in claim 17, which derivative is SakSTAR(S3C-MP5,E65D,K74R, E80A,D82A,K130T,K135R) (SY19(S3C-MP5)).

22. Staphylokinase derivative as claimed in claim 17, which derivative is SakSTAR(S3C-SP5,E65D,K74R, E80A,D82A,K130T,K135R) (SY19(S3C-SP5)).

23. Staphylokinase derivative as claimed in claim 17, which derivative is SakSTAR(S2C-SP5,S3C-SP5, E65D,K74R,E80A,D82A,K130T,K135R) (SY19(S2C-SP5,S3C-SP5)).

24. Staphylokinase derivative as claimed in claim 17, which derivative is SakSTAR(S3C-P20,E65D,K74R, E80A,D82A,K130T,K135R) (SY19(S3C-P20)).

25. Staphylokinase derivative as claimed in claim 17, which derivative is SakSTAR(S3C-P20,E65D,K74R, E80A,D82A,K130T,K135R) (SY19(S3C-P10)).

26. Dimer of two staphylokinase derivatives as claimed in claim 9.

27. Method for producing the staphylokinase derivatives as claimed in claims 1 to 8, comprising the steps of:

a. preparing a DNA fragment comprising at least the part of the coding sequence of staphylokinase that provides for its biological activity;

b. performing in vitro site-directed mutagenesis on the DNA fragment to replace one or more codons for wild-type amino acids by a codon for another amino acid;

c. cloning the mutated DNA fragment in a suitable vector;

d. transforming or transfecting a suitable host cell with the vector; and

e. culturing the host cell under conditions suitable for expressing the DNA fragment.

28. Method as claimed in claim 27, wherein the DNA fragment is a 453 bp EcoRI-HindIII fragment of the plasmid pMEX602sakB, the in vitro site-directed mutagenesis is performed and the mutated DNA fragment is expressed in *E. coli*.

29. Pharmaceutical composition comprising at least one of the staphylokinase derivatives as claimed in claims 1 to 25 together with a suitable excipient.

30. Pharmaceutical composition as claimed in claim 29 for treating arterial thrombosis.

CLAIMS

1. Staphylokinase derivatives showing a reduced immunogenicity as compared to wild-type staphylokinase, after administration to patients with arterial thrombosis.

2. Staphylokinase derivatives as claimed in claim 1 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by another amino acid thus reducing the reactivity with a panel of murine monoclonal antibodies.

3. Staphylokinase derivatives as claimed in claim 1 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by another amino acid thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase.

4. Staphylokinase derivatives as claimed in claim 1 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by other amino acids, without reducing the specific activity by more than 50 percent.

5. Staphylokinase derivatives SakSTAR(K35X, G36X,E65X,K74X,E80X,D82X,K102X,E108X,K109X,K121X,K130X, K135X,K136X,+137X) having the amino acid sequence as depicted in figure 1 in which one or more of the amino acids Lys in position 35, Gly in position 36, Glu in position 65, Lys in position 74, Glu in position 80, Asp in position 82, Lys in position 102, Glu in position 108, Lys in position 109, Lys in position 121, Lys in position 130, Lys in position 135 and/or Lys in position 136 have been replaced with other amino acids and/or in which one amino acid has been added at the COOH-terminus, thus altering the immunogenicity after administration in patients, without markedly reducing the specific activity.

6. Staphylokinase derivatives listed in Tabl s 1, 3, 4, 5, 6, 7, 8, 13, 19 and 20, having the amino acid sequence as depicted in figure 1 in which the indicated amino acids have been replaced by other amino acids thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase, without reducing the specific activity.

7. Staphylokinase derivative as claimed in claims 1-6 selected from the group consisting of SakSTAR(K74A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E75A), SakSTAR(E80A,D82A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(S34G,G36R, H43R), SakSTAR(K35A), SakSTAR(D82A), SakSTAR(D82A,S84A), SakSTAR(T90A), SakSTAR(Y92A), SakSTAR(K130A), SakSTAR(V132A), SakSTAR(S34G,G36R,H43R), SakSTAR(G36R), SakSTAR(H43R), SakSTAR(G36R,K74R), SakSTAR(K35E), SakSTAR(K74Q), SakSTAR(K130T), SakSTAR(V132L), SakSTAR(V132T), SakSTAR(V132N), SakSTAR(V132R), SakSTAR(K130T,K135R), SakSTAR(G36R,K130T,K135R), SakSTAR(K74R,K130T,K135R), SakSTAR(K74Q,K130T,K135R), SakSTAR(G36R,K74R,K130T,K135R), SakSTAR(G36R,K74Q, K130T,K135R), SakSTAR(G36R,H43R,K74R,K130T,K135R), SakSTAR(E65A,K74Q,K130T,K135R), SakSTAR(E65Q,K74Q, K130T,K135R), SakSTAR(K74Q,K86A,K130T,K135R), SakSTAR(E65Q,T71S,K74Q,K130T,K135R), SakSTAR(K74Q, K130A,K135R), SakSTAR(E65Q,K74Q,K130A,K135R), SakSTAR(K74Q,K130E,K135R), SakSTAR(K74Q,K130E, V132R,K135R), SakSTAR(E65Q,K74Q,T90A,K130A,K135R), SakSTAR(E65Q,K74Q,N95A,K130A,K135R), SakSTAR(E65Q,K74Q, E118A,K130A,K135R), SakSTAR(E65Q,K74Q,N95A,E118A,K130A, K135R), SakSTAR(N95A,K130A,K135R), SakSTAR(E65Q,K74Q, K109A,K130,K135R), SakSTAR(E65Q,K74Q,E108A,K109A, K130T,K135R), SakSTAR(E65Q,K74Q,K121A,K130T,K135R), SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,+137K), SakSTAR(E80A,D82A,K130T,K135R), SakSTAR(K74R,E80A,D82A, K130T,K135R), SakSTAR(K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A,K74R,E80A,D82A,K130T,K135R), SakSTAR(E65D, K74R,E80A,D82A,K130T,K135R), SakSTAR(E65S,K74R,E80A,

D82A,K130T,K135R), SakSTAR(S34G,G36R,K74R,K130T,K135R), SakSTAR(E65A,K74R,E80A,D82A,K130T,K135R), SakSTAR(E65N, K74R,E80A,D82A,K130T,K135R), SakSTAR(E65Q,K74R,E80A, D82A,K130T,K135R), SakSTAR(K57A,E58A,E61A,E80A,D82A, K130T,K135R), SakSTAR(E65D,K74Q,E80A,D82A,K130T,K135R), SakSTAR(E65Q,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A, E65D,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K74R,E80A,D82A, S103A,K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K109A, K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K130T, K135R,K136A), SakSTAR(E65Q,K74Q,D82A,S84A,K130T,K135R), SakSTAR(K35A,K74Q,E80A,D82A,K130T,K135R), SakSTAR(K35A, E65D,K74R,E80A,D82A,K130T,K135R).

8. SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) having the code SY19.

9. SakSTAR(K35A,E65Q,K74R,E80A,D82A,T90A,E99D, T101S,E108A,K109A,K130T,K135R) having the code SY161.

10. Staphylokinase derivatives as claimed in claims 1-9 having an amino acid substituted with Cys, resulting in dimerization and/or increased specific activity and/or reduced clearance and/or increased thrombolytic potency.

11. Staphylokinase derivatives as claimed in claims 1-10 with polyethylene glycol substitution, characterized by a maintained specific activity and a significantly reduced plasma clearance.

12. Staphylokinase derivatives as claimed in claim 10 wherein the Cys is chemically modified with polyethylene glycol with molecular weights up to 20 kDa.

13. Staphylokinase derivatives as claimed in claim 12 wherein selected amino acids in the NH_2-terminal region of 10 amino acids, are substituted with Cys, which is chemically modified with polyethylene glycol with molecular weights up to 20 kDa, which derivatives are characterized by a significantly reduced plasma clearance and maintained thrombolytic potency upon single intravenous bolus administration at a reduced dose.

14. Staphylokinase derivative as claimed in claim 13, wherein the serine in position 2 or 3 is

substituted with a cyst in and the cystein is chemically modified with polyethyl ne glycol having a molecular weight of 5, 10 or 20 kDa.

15. Staphylokinase derivative as claimed in claim 14, which derivative is SY161(S3C-MP5) as defined in table 20.

16. Staphylokinase derivative as claimed in claim 14, which derivative is SY161(S3C-P10) as defined in table 20.

17. Staphylokinase derivative as claimed in claim 14, which derivative is SY161(S3C-P20) as defined in table 20.

18. Staphylokinase derivative as claimed in claim 14, which derivative is SY19(S3C-MP5) as defined in table 20.

19. Staphylokinase derivative as claimed in claim 14, which derivative is SY19(S3C-SP5) as defined in table 20.

20. Staphylokinase derivative as claimed in claim 14, which derivative is SY19(S2C-SP5,S3C-SP5) as defined in table 20.

21. Staphylokinase derivative as claimed in claim 14, which derivative is SY19(S3C-P20) as defined in table 20.

22. Staphylokinase derivative as claimed in claim 14, which derivative is SY19(S3C-P10) as defined in table 20.

23. Dimer of two staphylokinase derivatives as claimed in claim 10.

24. Method for producing the staphylokinase derivatives as claimed in claims 1 to 10, comprising the steps of:

a. preparing a DNA fragment comprising at least the part of the coding sequence of staphylokinase that provides for its biological activity;

b. p rforming in vitro site-directed mutagenesis on the DNA fragment to r place ne or more

codons for wild-typ amin acids by a codon f r an ther amino acid;

c. cloning the mutated DNA fragment in a suitable vector;

d. transforming or transfecting a suitable host cell with the vector; and

e. culturing the host cell under conditions suitable for expressing the DNA fragment.

25. Method as claimed in claim 24, wherein the DNA fragment is a 453 bp EcoRI-HindIII fragment of the plasmid pMEX602sakB, the in vitro site-directed mutagenesis is performed and the mutated DNA fragment is expressed in E. coli.

26. Pharmaceutical composition comprising at least one of the staphylokinase derivatives as claimed in claims 1 to 23 together with a suitable excipient.

27. Pharmaceutical composition as claimed in claim 26 for treating arterial thrombosis.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rul s 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
L/UV24/102		
International application No. PCT/EP 99/00748	International filing date (*day/month/year*) 04/02/1999	(Earliest) Priority Date (*day/month/year*) 04/02/1998

Applicant

LEUVEN RESEARCH & DEVELOPMENT VZW et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 7 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

IDENTIFICATION, PRODUCTION AND USE OF STAPHYLOKINASE DERIVATIVES WITH REDUCED IMMUNOGENICITY AND /OR REDUCED CLEARANCE

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 3

[ ] as suggested by the applicant.

[X] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 99/00748

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 1-6, and in part 7,10-14,23-27
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1.1). Present claim 1 relate to staphylokinase derivatives defined by reference to a desirable characteristic or property, namely to staphylokinase derivatives showing a reduced immunogenicity as compared to wild-type staphylokinase, after administration to patients with arterial thrombosis.

The claims cover all staphylokinase derivatives having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

1.2). Present claims 2-6,10-14 relate to an extremely large number of possible staphylokinase derivatives, and claims 24 and 25 relate to an extremely large number of methods.
For instance, claims 2-4 relate to staphylokinase derivatives having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by antoher amino acid thus reducing the reactivity with a panel of murine monoclonal antibodies (claim 2), or thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase (claim 3), or without reducing the specific activity by more than 50 percent (claim 4).
Claim 6 relates to staphylokinase derivatives listed in Tables 1-8,13,19, and 20 having the amino acid sequence as depicted in figure 1 in which the indicated amino acids have been replaced by other amino acids thus reducing the absorption of SakSTAR-specific antibodies.. without reducing the specific acitivity.
The staphylokinase derivatives of claim 10 are the derivatives of claims 1-9 and, further, having an amino acid substituted with Cys, resulting in dimerization and/or increase specific acitivity and/or reduced clearance and/or increased thrombolytic potency.
The staphylokinase derivatives of claim 11 are the derivatives of claims 1-10 with polyethylene glycol (PEG) substitution, characterized by a maintained specific activity and a significantly reduced plasma clearance. A similar functional limitiation is given for claim 13.

In fact, the claims contain so many options and for the method claims so many possible mutated DNA fragments to be expressed that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible.
Moreover, the attention of the applicant is drawn to the fact that the further functional characterization (i.e.aim to be achieved) given within said claims 4-6,10,11, and 13 is not suitable to render the scope of said claims clear (Art. 6 PCT).

1.3). Present claim 7 relates to an extremely large number of possible staphylokinase derivatives. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the products claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

be supported and disclosed, namely those parts relating to the following staphylokinase derivatives or combination variants of SakSTAR and apparently having the desired properties, namely reduced immunogenicity and thrombolytic efficacy:

- SakSTAR (K74A,E75A,R77A),
- SakSTAR (E80A,D82A),
- SakSTAR (E75A),
- SakSTAR (K35A,E75A),
- SakSTAR (E80A),
- SakSTAR (D82A),
- SakSTAR (E75A,D82A),
- SakSTAR (K35A),
- SakSTAR (G36A),
- SakSTAR (K130A),
- SakSTAR (V132A),
- SakSTAR (K74Q),
- SakSTAR (K130T),
- SakSTAR (V132R),
- SakSTAR (K130T,K135R),
- SakSTAR (E65Q,K74Q,K130T,K135R),
- SakSTAR (E65A,K74Q,K130T,K135R),
- SakSTAR (E80A,D82A,K130T,K135R),
- SakSTAR (K74R,E80A,D82A,K130T,K135R),
- SakSTAR (K74Q,E80A,D82A,K130T,K135R),
- SakSTAR (E65D,K74Q,E80A,D82A,K130T,K135R),
- SakSTAR (K35A,E65D,K74Q,E80A,D82A,K130T,K135R),
- SakSTAR (E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,+137K),
- SakSTAR (E65D,K74R,E80A,D82A,K130T,K135R),
- SakSTAR (E65S,K74R,E80A,D82A,K130T,K135R),

1.4). The search has been carried out for staphylokinase derivatives having an amino acid substituted with Cys or with PEG substitution (claims 10-14), in so far as these derivatives relate back to the above specifically mentioned staphylokinase derivatives.
The above comment also applies for claims 23-27.

2). The search has been carried out for all of the above mentioned derivatives and variants although the present international application lacks in principle unity of invention, since certain of the above mentioned SakSTAR derivatives were already known from the prior art. Therefore,their exists no longer a technical relationship between the different staphylokinase derivatives of claim 7.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-6, and in part 7,10-14,23-27

..

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No
CT/EP 99/00748

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C07K14/31 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	COLLEN, D. ET AL: "Thrombolytic properties of poorly immunogenic variants of recombinant staphylokinase." FIBRINOLYSIS & PROTEOLYSIS, (JUNE, 1998) VOL. 12, NO. SUPPL. 1, PP. 30. MEETING INFO.: XIVTH INTERNATIONAL CONGRESS ON FIBRINOLYSIS AND THROMBOLYSIS LJUBLJANA, SLOVENIA JUNE 22-26, 1998 , XP002111034 abstract ---	7,8
X	COLLEN D ET AL: "Recombinant staphylokinase variants with altered immunoreactivity. III: Species variability of antibody binding patterns." CIRCULATION, (1997 JAN 21) 95 (2) 455-62. , XP002111035 page 456; tables 2,3 ---	7,23-27

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
2 August 1999	11.08.99
Name and mailing address of the ISA	**Authorized officer**
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COLLEN D ET AL: "Recombinant staphylokinase variants with altered immunoreactivity. II: Thrombolytic properties and antibody induction." CIRCULATION, (1996 JUL 15) 94 (2) 207-16. , XP002111036 page 214 - page 215	7,23-27
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Information on patent family members

International Application No
PCT/EP 99/00748

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